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# THE NATURE OF THE SUGAR IN FOUR CASES OF PENTOSURIA.*

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(*From the Littauer Pneumonia Research Fund, New York University, New York.*)

(Received for publication, May 17, 1930.)

Ever since the discovery of the first case of pentosuria by Salkowski and Jastrowitz (1) the nature of the pentose excreted has excited considerable interest. The first definite report was that of Neuberg (2) who stated that he had isolated *i*-arabinose. Because of this report and also because of the apparent optical inactivity of the pentose-containing urine, it has generally been believed that *i*-arabinose is present in all cases. However, more recent studies indicate that the apparent optical inactivity of the urine may have been due to the low concentration of the sugar and that, at least in a number of cases (3–10), the sugar was actually dextrorotatory.

In fact, only Neuberg, Cammidge and Howard (11), and possibly Aron (12) have isolated from the urine any derivatives of *i*-arabinose. All of the other reported attempts at the isolation of the pentose or of its derivatives have indicated that the sugar was probably *d*-xyloketose (8–10, 13). This identification was made quite certain by Levene and La Forge (9).

The present paper is a report of the isolation from the urine of four different individuals of an osazone and a *p*-bromophenylhydrazone having properties identical with those described by Levene and La Forge. A more extended study was made with the material obtained from the first of these cases. This included the liberation of the sugar from the *p*-bromophenylhydrazone, determination of its optical activity, and attempts at crystallization. The latter were unsuccessful as were, also, all attempts at the

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* Aided by a grant from the American Association for the Advancement of Science.

separation of the sugar from the urine without the use of the expensive *p*-bromophenylhydrazine.

All four individuals are Jews. One is a woman; the others, males. Case 1 has already been reported by Margolis (14). For Case 2, I am indebted to Dr. Herman Sharlit and for Cases 3 and 4 to Dr. Herman O. Mosenthal.

In view of the association of pentosuria with migraine by Margolis, it is interesting to observe that our Case 2 had periodic headaches between the ages of 8 and 18 and that these had disappeared shortly before the presence of sugar in the urine had been discovered, 3 years ago. Although they were questioned in regard to this, there is no history of headache in either of the other two individuals. They are now 40 and 20 years old respectively and the condition has been known to exist for 18 months in Case 3 and for 6 months in Case 4.

The ratio of sugar to nitrogen in the urine of all four cases was about the same, 1:3 or 4. The osazones, prepared in the usual manner, melted at 157–160°. When recrystallized with an equal weight of *l*-xylosazone, the melting point rose to 195–202°. The optical properties of the osazone were determined in the first case.

In spite of several attempts, it was not found possible to obtain *p*-bromophenylhydrazone from the urine of Case 1 by following the directions given by Levene and La Forge. This may have been due to the presence in the urine of foreign substances due to the ingestion of various sedatives by the patient. Several modifications of the method were tried and one was finally adopted. This was also employed upon the urine of the three other individuals. It is possible that the original method of Levene and La Forge might have been successful with these urine samples. It was not tried because only limited quantities of the urine were available.

The method depends upon the removal of phosphate ion and most of the sulfate and chloride ions with neutral lead acetate, precipitation of the sugar with basic lead acetate and sodium hydroxide (Plimmer and Skelton (15)), decomposition with carbon dioxide (Levene and La Forge), removal of most of the remaining nitrogenous constituents with $HgSO_4$ and $Ba(OH)_2$ (modified from West, Scharles, and Peterson (16)), concentration by means of alcohol, and final separation as the hydrazone.

10 liters of the urine are treated with a concentrated solution of neutral lead acetate until no more precipitate is produced. After standing about an hour, most of the liquid is decanted and filtered through a large Buchner filter. The precipitate is then transferred to the filter and sucked nearly dry. The suction is then discontinued and about a liter of water is placed in the funnel. This is allowed to stand overnight. Most of the water filters through, washing the precipitate. The suction is then again applied and the precipitate is pressed dry with the aid of a sheet of rubber, as described by Gortner (17). The filtrate is cooled to 5° in a freezing mixture and, while being stirred vigorously, a liter of a 25 per cent solution of basic lead acetate (Goulard's extract) is added. The cooling and stirring are continued while 40 per cent sodium hydroxide is added from a pipette or burette. About 100 cc. are required. Portions of the mixture are removed and filtered. The addition of the alkali is continued until further addition to the clear filtrate gives no precipitate. An excess of alkali must be avoided. At the correct point, the filtrate will give no precipitate upon the addition of a drop of sodium hydroxide solution and no color or only a trace upon the addition of thymolphthalein. The cold mixture is filtered upon two large Buchner funnels and pressed dry, a sheet of rubber again being used. This filtration requires about 4 hours. The precipitate is suspended in about 4000 cc. of water, preferably with the aid of a mechanical stirrer. About 200 gm. of solid carbon dioxide are then added and the stirring is continued. The decomposition of the precipitate is rapid and its completion is announced by the sudden foaming of the mixture. This can easily be controlled by the addition of a few drops of caprylic alcohol. The mixture is then acid to litmus. The stirring is continued for from 30 to 45 minutes longer and the mixture is then filtered on a large Buchner funnel. This material filters rapidly. The precipitate is pressed dry and is then suspended in about 2500 cc. of water and again stirred with carbon dioxide. After 30 minutes, or longer, the mixture is filtered and pressed dry. The filtrates are separately treated with $H_2S$ and are filtered, in order, upon the same fluted paper which is then washed with a little water. The filtrates are evaporated *in vacuo* to a volume of about 3000 cc. The nitrogen content is then determined upon 1 cc. portions, with 0.01 N acid and alkali. Enough

of a 30 per cent solution of $HgSO_4$ in 10 per cent $H_2SO_4$ to combine with all the nitrogen found, upon the assumption that each gm.-equivalent of nitrogen requires 1 mol of $HgSO_4$, is then added and the mixture is cooled by the addition of ice. A hot concentrated solution of $Ba(OH)_2$ is then added until the mixture is just neutral to litmus. It is then filtered, and the precipitate washed and pressed dry. The small amount of mercury in the filtrate is removed with $H_2S$ and the filtrate is evaporated *in vacuo* to about 75 cc. It is then slowly run into 1500 cc. of absolute alcohol, with constant stirring. After the addition of 150 cc. of anhydrous ether, the precipitate settles rapidly. It is filtered off and washed with a little absolute alcohol. The filtrate is again evaporated *in vacuo* to a volume of 35 or 40 cc. and the precipitation with alcohol is repeated. Only a small amount of precipitate is now formed. This is filtered out, the volume of the filtrate is measured, and 1 cc. is diluted to 10 or 25 cc. for a sugar determination by Sumner's method (18). The remainder is evaporated *in vacuo* to about 75 cc. and is then transferred to a glass evaporating dish. *p*-Bromophenylhydrazine in amount equal in weight to the indicated "glucose" content of the liquid is added and the mixture is stirred until it is all dissolved. Crystallization may begin in a few minutes but it is usually absent. The dish is placed in a vacuum desiccator over NaOH for 1 or 2 days. *Heating is unnecessary.* By that time, most of the alcohol has evaporated and a red oil which may or may not contain crystals is left. The dish is filled with cracked ice and water is slowly added. As the oil is mixed with the ice water, it sets to a mass of crystals. This is stirred to secure thorough mixture and, after an hour or two at 0°, is filtered and washed with ice water. The crystals are then extracted with alcohol-free ether until the filtrate is colorless. They are dried in a vacuum desiccator and recrystallized by solution in warm alcohol and precipitation with ice water. Extraction of the recrystallized material with ether generally removes a little more coloring matter. The yield is about 0.5 gm. of hydrazone for each gm. of "sugar" in the original urine. This is less than was obtained by Levene and La Forge.

The material obtained from Case 1 was identified by its melting point, decomposition point, and optical rotation. The other preparations of the hydrazone were identified merely by the melting and decomposition points, which remained unchanged when the material was mixed with an equal quantity from Case 1. The data are summarized in Table I and the following text.

## *Effect of Bromine upon the Urine Pentose.*

*Case 1.*—A solution of the partly purified sugar, before the treatment with alcohol, contained 3.68 mg. per cc. After treatment with bromine for 2 days, it contained 3.23 mg. per cc.

*Case 2.*—A solution of the partly purified sugar, after the treatment with alcohol, contained 10.4 mg. per cc. After treatment with bromine for 1 day, it contained 10.0 mg. per cc.

TABLE I.

*Data Concerning the Excretion and Properties of Pentose in Urine.*

| Case No. | Nitrogen. | Pentose.* | Phenylosazone. | | *p*-Bromophenylhydrazone. | |
|---|---|---|---|---|---|---|
| | | | From urine, melting point. | Mixed with *l*-xylosazone, melting point. | Melting point. | Decomposition point. |
| | *gm.* | *gm.* | *°C.* | *°C.* | *°C.* | *°C.* |
| 1 | 87.6† | 29.2 | 157–160 | 200–202 | 127–128 | 165 |
| 2 | 72.4 | 17.8 | 154–157 | 198–200 | 130–132 | 165 |
| 3 | 40.6‡ | 13.1 | 161–163 | 195–198 | 130–131 | 164 |
| 4 | 16.3§ | 4.6 | 156–158 | 202 | 130–132 | 165 |

* The reducing action was determined by Sumner's method (18) and the apparent glucose content calculated.

† See Margolis (14) for variations in the ratio of nitrogen to pentose excretion.

‡ 4600 cc.

§ 2600 cc.

## *Optical Activity of Phenylosazone.*

0.200 gm. of osazone from Case 1 was dissolved in a mixture of 4 cc. of pyridine and 6 cc. of methyl alcohol. Read in a 1 dm. tube, $[\alpha]$ in 10 minutes was 0.25°; after 48 hours, 0.60°.

## *Optical Activity of p-Bromophenylhydrazone.*

0.500 gm. of the hydrazone from Case 1 was dissolved in 5 cc. of alcohol. Read in a 1 dm. tube, $[\alpha]$ in 10 minutes was $-1.87°$; after 24 hours, $+2.43°$.

### *Decomposition of p-Bromophenylhydrazone.*

7.2 gm. of the hydrazone from Case 1 were decomposed with benzaldehyde according to the method described by Levene and La Forge. After extraction with ether, the aqueous solution was evaporated *in vacuo* and then diluted to 100 cc. In a 1 dm. tube, $[\alpha] = 1.18°$. Since the calculated pentose content was 3.39 gm., $[\alpha]_D^{22} = 34.8°$. Levene and La Forge give 33.15° but state that this value is to be regarded as a minimum.

The reducing action of the solution was determined by various methods. 1 mg. of the pentose was found to be the equivalent of 1.25 mg. of glucose by Sumner's method, 1.22 by Benedict's copper method (19), 1.45 mg. of glucose by Benedict and Osterberg's sodium hydroxide-picrate method (20), and 1.18 by their sodium carbonate-picrate method (21).

## SUMMARY.

The phenylosazone and the *p*-bromophenylhydrazone of the sugar have been isolated from the urine of four cases of pentosuria. Their properties are identical with those described by Levene and La Forge and indicate the sugar to be *d*-xyloketose.

The method of preparation of the *p*-bromophenylhydrazone has been modified.

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# VITAMINS IN DRIED FRUITS.

## II. THE EFFECT OF DRYING AND OF SULFUR DIOXIDE UPON THE VITAMIN A CONTENT OF FRUITS.*

By AGNES FAY MORGAN and ANNA FIELD.

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(Received for publication, April 16, 1930.)

Some of the early studies on the vitamin content of dried fruits and vegetables are open to the objection that the products studied either were not commercial or were dried under conditions that affect adversely many qualities other than vitamins of the products. This report covers a part of an investigation undertaken cooperatively by the two laboratories involved, and intended to show the effect on vitamins of some of the treatments ordinarily employed in successful drying. The inquiry has developed largely into a study of the conditions under which vitamins A and C are protected or destroyed. We have already reported the remarkably protective effect of sulfur dioxide (1) upon the vitamin C of dried peaches, and have continued the study of this factor in prunes, apricots, and figs. Sun drying with its long continued slow oxidations as compared with the relatively rapid dehydration process at higher temperatures might be expected to bring about a different rate of loss or retention in those vitamins, particularly A and C, which have been shown to be readily destroyed by oxidation. The protective effect of sulfur dioxide upon the vitamin C of peaches may be due either to the acid reaction thus induced or more probably to the reducing character of the sulfites formed. In either case a similar protection of vitamin A might be expected to be manifest in the same fruits. Evidence on this point is presented herein.

A recent publication by Cady and Luck (2) describes rather

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drastic treatment with sulfur dioxide of cod liver oil, butter fat, and alfalfa, with subsequent testing of these materials for vitamin A. Cod liver oil was found to have lost most of its vitamin A after treatment with sulfur dioxide for 15 minutes to 2 hours at 20–100°, but butter fat treated with the gas at 60° for 2 hours had lost but little, and alfalfa or spinach alcohol extracts treated with sulfur dioxide for 1 hour at 60° had apparently suffered no loss of vitamin A. The treated foods were incorporated in the basal diet, so that intake of the vitamin-containing materials was irregular. The difference between the effects upon cod liver oil and the other foods might well be due to autooxidation of the vitamin induced by the catalytic action of some constituent of the basal diet in the fashion observed by McCollum, Simmonds, and Becker (3) as due to ferrous sulfate, and by Mattill (4). Such catalysis might be conceived of as possible only in the presence of any sulfite compound formed by the sulfur dioxide treatment. Feeding of the foods to be tested in separate doses should obviate this uncertainty to some extent.

Since, as Anderegg and Nelson (5) have shown, water and ethyl alcohol tend to decrease the rate of autooxidation and unsaturated fatty acids to increase it, both butter and alcoholic extracts of alfalfa would be more resistant to such action than cod liver oil. It seems unnecessary, therefore, to postulate a difference in nature of the vitamin A of cod liver oil from that in butter and alfalfa to explain the observed difference in effect of sulfur dioxide when such striking oxidative differences have been shown to be due to the catalytic action of accompanying substances present in perhaps minute amount in the basal ration. The varying results obtained by Dulière, Morton, and Drummond (6), by von Euler, von Euler, and Karrer (7), Moore (8), and Hume and Smedley-Maclean (9) on the vitamin A activity of purified carotene crystals may be due to similar variations in the oxidative action of the fatty carriers of the carotene. The differences seen by Sherman, Quinn, Day, and Miller (10) between the amount of destruction of vitamin A in olive oil extracts of butter fat and of spinach when heated under anaerobic conditions may also be ascribed to possible differences in catalysts present rather than to differences in stability of the vitamin.

The vitamin A content of preserved food samples is no doubt determined partly by the catalytic effect of other substances naturally present in the foods as well as by the vitamin value of the fresh specimen. It is probably useless to expect, then, that similar preserving processes will yield products of like vitamin A retention from foods of different composition. As will be shown later, examples of such divergences were found among the fruits here reported upon.

*Preparation and Chemical Examination of Fruit.*[1]—All fruit samples, both fresh and dried, were gathered, dried, sealed, analyzed, and stored under the supervision of W. V. Cruess and P. F. Nichols of the Fruit Products Laboratory. Dried fruit samples were stored at 0°.

The peaches used were of the 1927 crop, Muir variety, grown near Walnut Creek, California, and were prepared by sulfuring, dehydration, or sun drying as previously described (1). The fresh fruit was ground and frozen. The prunes were of the 1928 crop, French variety, and were grown near Edenvale, California. The lye-dipped prunes were immersed for about 5 seconds in a practically boiling 0.5 per cent lye solution, followed by spraying with fresh water. The apricots, also of the 1928 crop, were of the royal variety and were grown near Watsonville, California. All dehydrated fruit samples were dried in a tunnel drier at temperatures increasing from about 49–71° as drying progressed, relative humidity similarly decreasing from 70 to 20 per cent, and with a constant air velocity of approximately 500 feet per minute. The time required for dehydration varied with the different fruits, ranging from 20 to 30 hours as shown in Table I.

The moisture content of the fresh fruit and of the dried peaches and apricots was determined by oven drying *in vacuo* at 70° for 12 hours, and of the dried prunes by the xylene distillation method. To allow for the presence of pits in the dried prunes an arbitrary adjustment of 1 per cent additional moisture was made.

The sulfur dioxide content of all dried samples was found by distillation with HCl into iodine, a modification of the official method of the Association of Official Agricultural Chemists (11).

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[1] The preparation and analysis of the samples were carried out by W. Y. Fong, P. F. Nichols, and H. M. Reed of the Fruit Products Laboratory, except that the moisture and sulfur dioxide determinations upon the dried prunes were made by R. S. Hiltner and B. E. Hatherell of the Dried Fruit Association of California. We are indebted to this Association also for the fruit and for other support of this investigation.

TABLE I.

*Preparation and Composition of Fruit.*

| Fruit. | Lot. | Method of preparation. | Moisture. | Net shrinkage. | pH | Sulfur dioxide. |
|---|---|---|---|---|---|---|
| | | | *per cent* | | | *parts per million* |
| Muir peaches, 1927 crop. | F | Cut, pitted, ground, sealed cold in 8 oz. tin containers, frozen and kept at −17°. | 79 5 | 1.00 | | |
| " " | D | Cut, pitted, sun-dried 8 days, dried in stack 6 days. | 19 7 | 3.93 | 4.2 | |
| " " | SD | Cut, pitted, sulfured overnight, sun-dried 8 days, dried in stack 6 days. | 15.4 | 4 14 | 3 6 | 1875 |
| " " | E | Cut, pitted, dried in dehydrator at 63° for 20 to 24 hrs. | 19.1 | 3.96 | | |
| " " | SE | Cut, pitted, sulfured overnight, dried in dehydrator at 63° for 20 to 24 hrs. | 16 0 | 4 11 | | 1840 |
| Elberta peaches, 1928 crop. | LS | Cut, pitted, ground, placed in small glass jars, evacuated, filled with carbon dioxide, frozen, and kept at −17°. | 85.8 | 1.00 | | |
| French prunes, 1928 crop. | 10 | Cut, pitted, ground, sealed cold in 8 oz. tin containers, frozen and kept at −17°. | 62.6 | 1.00 | 3 5 | |
| " " | 11 | Dehydrated whole for 30 hrs. at 72° average temperature for 30 hrs. after lye dipping, washing, and sulfuring overnight. | 19.0 (flesh). | 2.17 | 3.2 | 1980 |
| " " | 12 | Same as for 11, but without sulfuring. | 18.2 | 2.19 | 3.5 | |
| " " | 13 | Same as for 11, but without lye dipping. | 18.6 | 2.18 | 3.2 | 1020 |
| " " | 14 | Same as 13, but sun-dried for 7 days, held in stack for 7 days, instead of being dehydrated. | 19.8 | 2.15 | 3.1 | 1005 |
| " " | 15 | Dehydrated whole for 30 hrs., without previous lye dipping or sulfuring. | 21.4 | 2.10 | 3.3 | |

TABLE I—*Concluded.*

| Fruit. | Lot. | Method of preparation. | Moisture. | Net shrinkage. | pH | Sulfur dioxide. |
|---|---|---|---|---|---|---|
| | | | *per cent* | | | *parts per million* |
| French prunes, 1928 crop. | 16 | Sun-dried 7 days, kept in stack 7 days, without previous lye dipping or sulfuring. | 20.2 | 2.13 | 3.6 | |
| “ “ | 17 | Lye-dipped, washed, sulfured overnight, sun-dried 7 days; held in stack 7 days. | 17.8 | 2.20 | 2.6 | 2695 |
| “ “ | 18 | Same as 17 except that sulfuring was omitted. | 17.0 | 2.22 | 3.2 | |
| Royal apricots, 1928 crop. | 5 | Pitted, ground, sealed cold in 8 oz. tin containers, frozen, and kept at −17°. | 82.9 | 1.00 | 3.3 | |
| “ “ | LS | Another fresh sample. Pitted, ground, placed in small glass jars, evacuated, filled with carbon dioxide, frozen, and kept at −17°. | 82.0 | 1.00 | | |
| “ “ | 1 | Cut, pitted, sulfured 3½ hrs., dehydrated 2 hrs. at 72° average temperature. | 18.8 | 4.75 | 2.4 | 515 |
| “ “ | 2 | Cut, pitted, sulfured 30 min., dehydrated 24 hrs. at 72° average temperature. | 17.0 | 4.85 | 2.9 | 125 |
| “ “ | 3 | Cut, pitted, dehydrated 24 hrs. at 72° average temperature. | 22.6 | 4.53 | 3.2 | 100 |
| “ “ | 4 | Cut, pitted, steamed 3½ min., sulfured 20 min., dehydrated 24 hrs. at 72° average temperature | 16.3 | 4.89 | 3.2 | 80 |
| “ “ | 6 | Cut, pitted, sulfured 2½ hrs., sun-dried 11 days. | 19.0 | 4.73 | 3.3 | 700 |
| “ “ | 7 | Same as 6, but sulfured only 30 min. | 17.5 | 4.82 | 3.1 | 470 |

The pH of fresh and rehydrated dry samples was determined by the hydrogen electrode upon the pulp. As may be noted in Table I, sulfuring usually, but not invariably, decreased the pH of the fruit.

Certain vitamin A values for 1928 Elberta peaches and royal apricots obtained by Laura Lee W. Smith in this laboratory upon samples grown at Davis, California, are included among our results for purposes of comparison. These samples were both of the fresh frozen type and differed from the others reported in that they were sealed in small jars, prepared by evacuation and release with carbon dioxide before being frozen in order to decrease the danger of oxidation.

*Methods of Testing for Vitamin A.*—The methods of testing for vitamin A used in this study were not markedly different from those which have now become usual for quantitative comparisons of vitamin content of foods. Rats from our own colony, reared by mothers fed a constant stock diet which had been used for several generations of animals, were placed at 21 days of age upon a vitamin A-free basal diet. This diet consisted of baked and alcohol-extracted casein 18 parts, irradiated Crisco 5, agar 2, salt mixture[2] 4, dextrin 71. In addition, 0.5 gm. of dried yeast was given separately to each rat daily. The fruit doses were weighed out accurately, made into pellets with a little of the basal ration and fed separately. In most cases the fruit was fed in three or four doses per week, although in a few cases the entire amount for the 56 days was given in one or two doses at the beginning of the period. The latter procedure was found to be undesirable, however, and to give less favorable findings than the usual daily or triweekly feeding.

The feeding of curative fruit doses was begun only after the usual signs of vitamin A deficiency had become evident. The period required for the production of these symptoms varied from 25 to 35 days. Ophthalmia occurred in practically all cases. The fruit feeding period was 56 days in length, and an attempt was made to obtain graded growth response to graded dosage. The standard unit chosen was that amount of fruit which produced an average weekly increase of weight of 6 to 8 gm. for the 8 weeks of observation. This rate of growth was chosen rather than the 3 gm. weekly advocated by Sherman and Munsell (12) because rats maintained at the latter rate are apt to be seriously affected by intercurrent and accidental results of their extremely low vitamin A margin.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 37, 572 (1919).

Postmortem examinations of all animals were made, particularly as to the condition of the stomach, kidneys, throat, and ears.

TABLE II.

*Vitamin A in Fresh and Dried Peaches.*

| Variety. | Lot. | Amount given daily. | Equivalent in fresh fruit. | Average weights. | | Average gain per wk. for 8 wks. | No. of rats used. | Condition of eyes. | Minimum dose, equivalent in fresh fruit allowing 6 gm. growth per wk. | Vitamin A retention in product. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Initial. | Final. | | | | | |
| | | *mg.* | *mg.* | *gm.* | *gm.* | *gm.* | | | *mg.* | *per cent* |
| Fresh Muir peaches, 1927 crop. | F | 100 | 100 | 47 | 88 | 5.1 | 4 | 1 ophthalmic. | 160 | 100 |
| | | 160 | 160 | 49 | 100 | 6.4 | 4 | Normal. | | |
| | | 200 | 200 | 107 | 173 | 8.2 | 2 | " | | |
| Unsulfured, dehydrated Muir peaches. | E | 34 | 135 | 104 | 95 | −1.0 | 2 | 1 ophthalmic. | >178 | About 50. |
| | | 40 | 158 | 69 | 96 | 3.3 | 3 | 2 " | | |
| | | 45 | 178 | 59 | 92 | 4 1 | 4 | 1 " | | |
| Unsulfured, sun-dried Muir peaches. | D | 34 | 133 | 105 | 151 | 5.7 | 2 | 1 ophthalmic. | 177 | 90 |
| | | 40 | 157 | 38 | 72 | 4.5 | 2 | 1 " | | |
| | | 45 | 177 | 54 | 114 | 7.5 | 4 | Normal. | | |
| Sulfured, dehydrated Muir peaches. | SE | 34 | 140 | 110 | 167 | 7.1 | 2 | Normal. | 140 | 100 |
| | | 40 | 164 | 62 | 117 | 6.9 | 3 | " | | |
| | | 45 | 185 | 72 | 126 | 6.8 | 4 | " | | |
| Sulfured, sun-dried Muir peaches. | SD | 34 | 141 | 156 | 139 | −2.0 | 1 | 1 ophthalmic. | 186 | 86 |
| | | 40 | 165 | 51 | 89 | 4.7 | 3 | Normal. | | |
| | | 45 | 186 | 50 | 100 | 6.2 | 2 | " | | |
| Fresh Elberta peaches, 1928 crop. | LS | 50 | 50 | 40 | 76 | 4.5 | 4 | Normal. | 100 | 100 |
| | | 100 | 100 | 50 | 107 | 7.1 | 4 | " | | |
| | | 200 | 200 | 46 | 116 | 8.7 | 4 | " | | |

Abscesses in throat and ears were commonly found in the rats which had died of the deficiency. Abnormalities in the kidneys

TABLE III.

*Vitamin A in Fresh and Dried Prunes.*

| French prunes, 1928 crop. | Lot No. | Amount fed daily | Equivalent in fresh fruit. | No. of rats used. | Condition of eyes. | Average weights. |  | Average gain per wk. for 8 wks. | Minimum daily dose to provide 6 gm. gain per wk. | Retention of vitamin A of fresh fruit. |
|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  |  | Initial. | Final. |  |  |  |
|  |  | *mg* | *mg.* |  |  | *gm.* | *gm.* | *gm.* | *mg.* | *per cent* |
| Fresh. | 10 | 100 | 100 | 2 | Normal. | 81 | 145 | 8.0 | 100 | 100 |
|  |  | 150 | 150 | 4 | " | 76 | 153 | 9.4 |  |  |
|  |  | 200 | 200 | 2 | " | 67 | 128 | 7.6 |  |  |
|  |  | 250 | 250 | 2 | " | 98 | 169 | 9 1 |  |  |
|  |  | 300 | 300 | 2 | " | 63 | 148 | 10.6 |  |  |
| Lye-dipped, sulfured, dehydrated. | 11 | 40 | 87 | 2 | 1 ophthalmic. | 94 | 123 | 3.4 | 109 | 91 |
|  |  | 40 | 109 | 3 | 1 " | 69 | 117 | 6 0 |  |  |
|  |  | 75 | 163 | 3 | 1 " | 79 | 132 | 6 8 |  |  |
| Lye-dipped, unsulfured, dehydrated. | 12 | 50 | 110 | 5 | 3 ophthalmic. | 89 | 114 | 3.3 | 240 | 41 |
|  |  | 75 | 164 | 3 | Normal. | 84 | 118 | 4 3 |  |  |
|  |  | 100 | 220 | 3 | 1 ophthalmic. | 94 | 135 | 5.0 |  |  |
|  |  | 110 | 240 | 4 | 2 " | 86 | 138 | 6 5 |  |  |
| Not lye-dipped, sulfured, dehydrated. | 13 | 50 | 110 | 6 | Normal. | 94 | 143 | 6 1 | 110 | 91 |
|  |  | 75 | 163 | 6 | 1 ophthalmic. | 94 | 143 | 6 1 |  |  |
| Not lye-dipped, sulfured, sun-dried. | 14 | 50 | 108 | 3 | Normal. | 93 | 103 | 1 2 | 161 | 62 |
|  |  | 75 | 161 | 8 | 2 ophthalmic. | 80 | 130 | 6 2 |  |  |
| Not lye-dipped, unsulfured, dehydrated. | 15 | 50 | 105 | 2 | 2 ophthalmic. | 91 | 76 | −1 7 | 420 | 24 |
|  |  | 75 | 160 | 5 | 4 " | 93 | 106 | 1 6 |  |  |
|  |  | 100 | 210 | 3 | 2 " | 88 | 92 | 0.5 |  |  |
|  |  | 200 | 420 | 5 | Normal. | 86 | 149 | 7.9 |  |  |
| Not lye-dipped, unsulfured, sun-dried. | 16 | 50 | 107 | 3 | 2 ophthalmic. | 78 | 105 | 3.4 | 232 | 43 |
|  |  | 75 | 160 | 3 | 1 " | 71 | 104 | 4.1 |  |  |
|  |  | 100 | 213 | 2 | 1 " | 104 | 149 | 5.6 |  |  |
|  |  | 110 | 232 | 5 | 1 " | 80 | 138 | 7.2 |  |  |
|  |  | 120 | 252 | 4 | 1 " | 93 | 151 | 7.2 |  |  |

TABLE III—*Concluded.*

| French prunes, 1928 crop. | Lot No. | Amount fed daily. | Equivalent in fresh fruit. | No. of rats used. | Condition of eyes. | Average weights. |  | Average gain per wk. for 8 wks. | Minimum daily dose to provide 6 gm. gain per wk. | Retention of vitamin A of fresh fruit. |
|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  |  | Initial. | Final. |  |  |  |
|  |  | *mg.* | *mg.* |  |  | *gm.* | *gm.* | *gm.* | *mg.* | *per cent* |
| Lye-dipped, | 17 | 50 | 110 | 4 | 2 ophthalmic. | 107 | 124 | 2 1 | 165 | 60 |
| sulfured, |  | 75 | 165 | 5 | 1 " | 89 | 143 | 6.8 |  |  |
| sun-dried. |  | 100 | 225 | 1 | Normal. | 89 | 154 | 8.1 |  |  |
| Lye-dipped, | 18 | 50 | 111 | 4 | 2 ophthalmic. | 85 | 115 | 3.7 | 222 | 45 |
| unsulfured, |  | 75 | 167 | 4 | 2 " | 92 | 139 | 5.7 |  |  |
| sun-dried. |  | 80 | 176 | 4 | Normal. | 81 | 126 | 5.7 |  |  |
|  |  | 85 | 188 | 3 | " | 83 | 128 | 5.6 |  |  |
|  |  | 100 | 222 | 4 | 1 ophthalmic. | 74 | 134 | 7.5 |  |  |

and crater-like tumors in the stomachs were also present in the vitamin A-low cases and occasionally even in those which had apparently resumed nearly normal growth. The persistence of slight ophthalmias was seen likewise in a few cases which had otherwise all indications of vitamin A normality.

The method of estimating per cent retention of vitamin A used in Tables II to IV, is somewhat arbitrary, since it is based upon the assumption that average increases in body weight are correlated with similar vitamin A intakes. The dose of the frozen fresh fruit which permits at least 6 gm. gain per week for 8 weeks is taken as the vitamin A unit and is considered to represent 100 per cent of the vitamin A content of this fruit. The dose of the dried fruit product, calculated as its fresh fruit equivalent, which allows a similar gain in weight is looked upon as containing a similar amount of vitamin A, and the ratio between the weight of the frozen fresh fruit dose and that of the dried product equivalent in fresh weight is taken as the ratio of loss of vitamin A. The resulting percentages are only roughly indicative of the changes which have occurred and are reliable only when consistently graded growth has been obtained from graded doses of the fruit.

TABLE IV.

*Vitamin A in Fresh and Dried Apricots.*

| Royal apricots, 1928 crop. | Lot No. | Amount fed daily. | Equivalent in fresh fruit. | No. of rats used. | Condition of eyes. | Average weights. | | Average gain per wk. for 8 wks. | Minimum daily dose to provide 6 gm. gain per wk. | Retention of vitamin A of fresh fruit. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Initial. | Final. | | | |
| | | *mg.* | *mg.* | | | *gm.* | *gm.* | *gm.* | *mg.* | *per cent* |
| Fresh. | 5 | 25 | 25 | 3 | Normal. | 92 | 151 | 7.3 | 25 | 100 |
| | | 50 | 50 | 5 | " | 66 | 144 | 9.7 | | |
| | | 100 | 100 | 4 | " | 60 | 131 | 8.6 | | |
| | | 150 | 150 | 3 | " | 68 | 149 | 10.2 | | |
| Sulfured, dehydrated. | 1 | 10 | 48 | 2 | 1 ophthalmic. | 53 | 75 | 2.6 | 95 | 26 |
| | | 20 | 95 | 4 | Normal. | 58 | 110 | 6.5 | | |
| | | 30 | 143 | 2 | " | 51 | 111 | 7.5 | | |
| Sulfured, dehydrated. | 2 | 5 | 24 | 2 | 1 ophthalmic. | 73 | 99 | 3.3 | 49 | 51 |
| | | 10 | 49 | 4 | Normal. | 63 | 113 | 6.3 | | |
| | | 20 | 97 | 3 | " | 60 | 117 | 7.1 | | |
| | | 30 | 146 | 2 | " | 70 | 128 | 7.2 | | |
| Unsulfured, dehydrated. | 3 | 10 | 45 | 2 | 1 ophthalmic. | 68 | 101 | 4.1 | 146 | 16 |
| | | 20 | 90 | 4 | Normal. | 65 | 97 | 4.0 | | |
| | | 30 | 135 | 5 | 2 ophthalmic. | 86 | 127 | 5 1 | | |
| | | 35 | 157 | 4 | 1 " | 89 | 159 | 8.7 | | |
| | | 40 | 170 | 2 | 1 " | 72 | 149 | 9 6 | | |
| Sulfured, dehydrated, steamed 3½ min. | 4 | 10 | 49 | 4 | Normal. | 68 | 118 | 6.2 | 123 | 20 |
| | | 20 | 98 | 9 | " | 71 | 110 | 5.0 | | |
| | | 25 | 123 | 3 | " | 86 | 139 | 6.6 | | |
| | | 30 | 147 | 3 | 1 ophthalmic. | 73 | 140 | 8.3 | | |
| Sulfured, sun-dried. | 6 | 10 | 47 | 3 | 1 ophthalmic. | 81 | 99 | 2.2 | 118 | 21 |
| | | 20 | 95 | 4 | Normal. | 62 | 106 | 5.5 | | |
| | | 25 | 118 | 4 | 1 ophthalmic. | 87 | 151 | 7.8 | | |
| | | 30 | 142 | 2 | Normal. | 58 | 117 | 7.3 | | |
| Sulfured, sun-dried. | 7 | 10 | 48 | 3 | Normal. | 62 | 80 | 2.3 | 144 | 16 |
| | | 20 | 96 | 4 | 1 ophthalmic. | 76 | 111 | 4.4 | | |
| | | 30 | 144 | 5 | Normal. | 79 | 130 | 6 4 | | |
| | | 40 | 192 | 2 | " | 68 | 153 | 10.6 | | |
| Fresh (another sample). | LS | 50 | 50 | 4 | Normal. | 52 | 119 | 8.4 | <50 | 100 |
| | | 100 | 100 | 4 | " | 82 | 164 | 10.2 | | |
| | | 200 | 200 | 3 | " | 79 | 181 | 12.8 | | |

## DISCUSSION.

*Peaches.*—The sulfured peach products, as shown in Table II, both dehydrated and sun-dried, appeared to contain almost the whole, 86 to 100 per cent, of the vitamin A present in the corresponding fresh fruit. The unsulfured sun-dried fruit likewise retained about 90 per cent of the vitamin, but the unsulfured dehydrated product appeared to have lost nearly 50 per cent. This latter figure is only approximate since a large enough dose was not given to obtain the standard minimum weight increase of 6 gm. per rat per week. The same relation was observed in the corresponding prune preparations. Of course, the higher temperatures used in dehydration may in the absence of the protecting sulfur dioxide have proved more destructive than the sun drying temperatures.

There is apparently little danger of loss of the vitamin A of yellow peaches by either sun drying or dehydration, just as there is little loss of vitamin C in the sulfured peaches (1). The reason for this immunity to the usual loss from oxidation may reside in a low oxidase content of the fruit or in the absence of other autooxidative catalysts.

It is interesting to note the correspondence between the amount of yellow pigment and of vitamin A present in the fresh peaches. The Muir peaches (Lot F) were a rather pale yellow in color as compared with the deep orange-yellow of the Elberta peaches (Lot LS), and the vitamin A content of the latter as found by Laura Lee W. Smith in this laboratory is definitely greater. White peaches preserved and tested similarly were found to be almost wholly lacking in the vitamin. These findings are in line with the recent claims of von Euler, von Euler, and Karrer (7) and of Moore (8) that purified carotene is effective in minute quantities as a source of vitamin A.

*Prunes.*—It is clear that sulfur dioxide is of some value in preserving the vitamin A of prunes, for the two sulfured varieties in the dehydrated (Lots 11, 13) and in the sun-dried (Lots 14, 17) products show higher retentions than do the unsulfured fruit. These results are shown in Table III. But of even more importance is the choice of dehydration instead of sun drying if sulfuring is used, since both dehydrated sulfured products retain 91 per cent of the vitamin A as compared with 60 and 62 per cent retained by the sun-dried sulfured fruit. However, again as in the peaches, when sulfuring is not used, sun drying leaves more vitamin A in the fruit, 57 and 45 per cent, than does dehydration, 41 and 24 per cent.

The effect of the lye dipping of prunes, which is the common commercial practice, upon vitamin A retention is not marked, although the dipped unsulfured prunes appear to have a higher content of the vitamin than do the corresponding undipped products. This is not the same as was found with regard to vitamin C retention,[3] for the undipped sulfured prunes were found to have lost more of their original antiscorbutic property than did the lye-dipped sulfured fruit. Since all unsulfured fruit was without vitamin C value, the effect of the dipping in those cases was indiscernible. Apparently the lye dipping promoted vitamin C retention, through its effect upon the sulfuring, since the cracked surface of the dipped fruit absorbed more sulfur dioxide than did that of the undipped fruit. This is evident from a comparison of the figures for sulfur dioxide in the four sulfured products, Lots 11, 13, 14, and 17. The dipped prunes, Lots 11 and 17, show 2 to 3 times as much sulfur dioxide as do the corresponding undipped prunes, Lots 13 and 14. But the order of vitamin A retention is Lots 11, 13, 14, and 17, instead of Lots 17, 11, 13, and 14, as it should be if sulfur dioxide retention alone governed the vitamin A protection in the same fashion that it governs vitamin C protection.

As shown in Table V, sulfured dehydrated prunes have almost all of the vitamin A of the fresh fruit, sun-dried sulfured prunes about 60 per cent, and unsulfured prunes both dehydrated and sun-dried, 45 to 24 per cent.

The pH of the prune and apricot samples varied surprizingly little, the sulfured samples showing, as might be expected, slightly lower figures, and Lots 17 and 1, which were most highly sulfured, the lowest of all.

*Apricots.*—As shown in Table IV, an astonishingly large amount of the vitamin is present in fresh apricots and in all dried apricots, even though a large loss of vitamin A occurs in the dried products.

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[3] Results to be published later.

Again the sulfured products seem to retain more than do the unsulfured, but the actual amount of sulfur dioxide present in the fruit appears not to be the governing factor. The unusual content of vitamin A in apricots is of interest in connection with the excellent hemoglobin regeneration produced by this fruit, as observed by Robscheit-Robbins and Whipple (13).

TABLE V.

*Summary of Vitamin A in Dried Fruits.*

| Fruit. | Lot. | Method of preparation. | | | Vitamin A of fresh fruit retained. |
|---|---|---|---|---|---|
| | | Sun drying or dehydrating. | Sulfur dioxide. | Lye dipping. | |
| | | | *parts per million* | | *per cent* |
| Peaches. | SE | Dehydrated. | 1840 | | 100 |
| | D | Sun-dried. | Unsulfured. | | 90 |
| | SD | “ | 1875 | | 86 |
| | E | Dehydrated. | Unsulfured. | | 50 (about). |
| Prunes. | 11 | Dehydrated. | 1980 | Dipped. | 91 |
| | 13 | “ | 1020 | Not dipped. | 91 |
| | 14 | Sun-dried. | 1005 | “ “ | 62 |
| | 17 | “ | 2695 | Dipped. | 60 |
| | 18 | “ | Unsulfured. | “ | 45 |
| | 16 | “ | “ | Not dipped. | 43 |
| | 12 | Dehydrated. | “ | Dipped. | 41 |
| | 15 | “ | “ | Not dipped. | 24 |
| Apricots. | 2 | Dehydrated. | 125 | | 51 |
| | 1 | “ | 515 | | 26 |
| | 4 | “<br>(steamed). | 80 | | 20 |
| | 6 | Sun-dried. | 700 | | 21 |
| | 7 | “ | 470 | | 16 |
| | 3 | Dehydrated. | 100<br>(unsulfured). | | 16 |

The quantities of sulfur dioxide found in the apricot samples were lower than were expected, particularly in the samples which were thought to be heavily sulfured. None of the apricots was sulfured overnight as were the peaches and prunes, and consequently less sulfur dioxide was retained by the former fruit. Sulfuring periods of 20 minutes to 3½ hours are long enough to produce satisfactorily bleached apricots but apparently not long enough to insure complete protection of vitamin A. Similar results were encountered in the tests for vitamin C in this fruit. The discrepancy between the retention of vitamin A in Lots 1 and 2 is surprizing since amount of sulfuring and pH of the former product would lead one to expect it to be superior instead of inferior to the latter. The difference may not be significant, however.

Sun drying involves greater loss than does dehydration when the sulfuring process is used. Since the one unsulfured apricot product, Lot 3, happened to be dehydrated, it is impossible to say whether the curious exception to the superiority of dehydration in unsulfured fruit noted in the peaches and prunes is true of apricots as well.

The losses of 49 to 84 per cent of the vitamin in the drying of apricots may well be connected with the unusual air retention seen in the frozen fresh fruit. As will be shown in a later report, the fresh frozen apricots lost much of their antiscorbutic property when frozen without previous evacuation and at the same time on thawing gave off a considerable amount of gas, apparently largely air. Similar losses were not encountered in the frozen fruit used for vitamin A determination even when compared with the results of determinations upon royal apricots, Lot LS, frozen after evacuation. This might be taken to indicate less susceptibility to autooxidation by the vitamin A than the vitamin C, or perhaps the presence in this fruit of catalysts peculiarly active in regard to the latter vitamin.

The possible rôle of the ultra-violet portion of sunlight in promoting oxidations of both vitamins A and C must be considered in evaluating the effects of sun drying. The antioxidative effect of the sulfur dioxide appears to function under these circumstances, however, since the sun-dried products were richer in vitamin A than were the dehydrated ones without sulfur, but the opposite was true when sulfur was used.

*Effect of Storage.*—After 12 to 14 months storage at 0° several of the dried prune and apricot products were retested in order to fill in the series of graded dosages. The products thus examined were Lots 12, 15, 16, and 18 of the prunes and 3 and 4 of the apricots. As may be seen in Tables III and IV, the results fell into line quite acceptably with the results recorded at least a year previously upon the same samples. The prune doses used in the second series were 110 mg. of Lot 12, 200 mg. of Lot 15, 110 and

120 mg. of Lot 16, and 80 and 85 mg. of Lot 18. The apricot doses were 35 mg. of Lot 3 and 25 mg. of Lot 4. It is fair to assume probably that no losses of vitamin A are likely to occur on long storage of these dried fruits at 0°.

The actual quantity of vitamin A in the frozen fresh apricots is surprizingly large. Results obtained by the same methods (14) in this laboratory appear to show that the apricots are 4 or 5 times as active as tomatoes and to compare favorably with such rich sources of the vitamin as egg yolk, butter, and spinach (15), even when the values of the latter are expressed in terms of the smaller unit, 3 gm. growth per rat per week, used by Sherman and Munsell (12). In consequence, even the least successful of the drying processes left a good proportion of vitamin A in the apricot products.

The yellow peaches are about one-fourth as rich in vitamin A as are the apricots and in the case of the Elberta variety appear to be almost identical in value with tomatoes. The paler Muir peach has about two-thirds the vitamin A value of the Elberta. The dried peach products on the other hand proved to be almost as rich in the vitamin as the dried apricots because of their almost perfect protection of the vitamin during the drying processes.

The prunes, contrary to our earlier expectations, appeared to be fully equal to the Elberta peaches in vitamin A value. Tests upon this fruit are being made to discover the carotenoid content of the pulp in pursuance of the question as to the identity of carotene and vitamin A.

Compared with tomatoes (14) tested by the same criteria, and bananas (16) and lettuce (17) by the less severe standards of Sherman and Munsell, both Elberta and Muir peaches and French prunes are considerably the richer sources of vitamin A.

It is interesting to note that the conditions which have been found to favor retention of both vitamins A and C in dried fruit products are just those which popular prejudice and even official regulation have heretofore frowned upon. Indeed, there are to be found among so called "health foods" now on the market, high priced special preparations of sun-dried and unsulfured fruits. The sulfured dehydrated fruits on the other hand, have here been found without exception to be superior in vitamin retention.

## SUMMARY.

1. The vitamin A content of frozen fresh and variously dried samples of peaches, prunes, and apricots was determined by uniform biological technique.

2. *The sulfured dehydrated* fruit in all cases appeared to retain the largest proportion of the vitamin, but this retention was not found to be directly related to the amount of sulfur dioxide in the fruit.

3. Of the *unsulfured* fruit, the *sun-dried* specimens of prunes and peaches showed better retention of vitamin A than did the corresponding *unsulfured dehydrated* products. The more destructive effect, in the absence of the protective sulfur dioxide, of the higher temperatures used in dehydration is the only explanation of this divergence offered.

4. *Lye dipping* of the dried prunes seemed to exert no effect upon vitamin A retention.

5. The amount of destruction of vitamin A produced by comparable methods of preservation varies widely in the three fruits.

The vitamin A of *peaches* seems little affected by any of the drying processes, 86 to 100 per cent being retained in all cases. The vitamin A of *prunes* is more labile, 24 to 91 per cent being retained, and that of *apricots* still more easily affected, only 16 to 51 per cent of the fresh fruit value being present in the dried preparations. However, the dried apricots which had lost the greatest proportions of their fresh fruit vitamin A content were still absolutely richer in this vitamin than the best of the peach and prune products. Differences in amounts and kinds of oxidative catalysts present in the fruits are thought to account for these variations.

6. Storage of both sulfured and unsulfured apricots and prunes at 0° for a period of more than a year brought about no detectable loss of vitamin A content.

7. The vitamin A content of two varieties of yellow peaches, of prunes, and of apricots is shown to be relatively large, that of the apricots comparing favorably with the best figures reported for spinach, egg yolk, or butter. The peaches and prunes had less vitamin A than the apricots but as much or more than tomatoes, bananas, or lettuce.

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# ON WALDEN INVERSION.

## XIV. THE INFLUENCE OF SUBSTITUTING GROUPS ON OPTICAL ROTATION IN THE SERIES OF DISUBSTITUTED ACETIC ACIDS CONTAINING A PHENYL GROUP.

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In a previous communication (1) we discussed the influence of substitution in a series of disubstituted acetic acid derivatives of the aliphatic series. In this series each substance contained only one group of significant polarity. In the present paper observations are presented on the derivatives of disubstituted acetic acids in which one of the substituting groups is aromatic, hence contains an additional polar group. The observations on this series of derivatives are of special importance as an aid for the interpretation of observations previously made on substitutions in secondary carbinols of mixed aliphatic-aromatic character. The substitution of the hydroxyl group by a halogen in the carbinols of that class is, under certain conditions, accompanied by Walden inversion. To be able to correlate configurations of the carbinols and halides is of fundamental importance for the purpose of singling out the cases in which a reaction of substitution is accompanied by Walden inversion.

In the case of the derivatives of dextro-disubstituted aliphatic acetic acid, the values of rotations descended with the changes in polarities of the substituting groups in the following order,

$$C{\equiv}N > COOC_2H_5 > COOH > CONH_2 > COCl > CH_2SO_3H > CH_2X >$$
$$CH_2SH > CH_2OH > CH_2NH_2$$

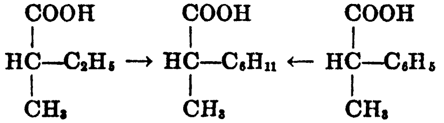
the direction of rotation being turned to the left for the carbinols and for the amines. It is self-evident that for the levorotatory series the changes of rotation should be of descending numerical value, becoming dextrorotatory for the carbinols and for the amines.

The events in the mixed aliphatic-aromatic series are markedly different. If the rotations of the first member of the series, namely dextro-phenylmethylacetic acid, and of the more significant derivatives of it are compared, the following descending order is observed.

$$\text{—CO}\overset{+}{\text{O}}\text{H} > \text{—CH}_2\overset{+}{\text{O}}\text{H} > \text{—C}\overset{-}{\text{H}_2}\text{X} > \text{—C}\equiv\text{N}$$

I.

Comparing these changes in rotations with those observed in the same derivatives of the dextro acids of the aliphatic series, one finds the order of change for $—\text{C} \equiv \text{N}$, $\text{CH}_2\text{X}$, $\text{CH}_2\text{OH}$, reversed. The question arises whether the two acids and their derivatives are configurationally of similar or of opposite series. With some degree of probability this question can be answered by hydrogenating dextro-phenylmethylacetic acid to the cyclohexylmethylacetic acid. This change brought about a significant drop in the value of rotation but did not alter the direction of rotation. On the assumption then that dextro-phenylmethylacetic acid has an analogous configuration to dextro-ethylmethylacetic acid, which might provisionally be given by the following figure



$$\begin{array}{ccccc} \text{COOH} & & \text{COOH} & & \text{COOH} \\ | & & | & & | \\ \text{HC—C}_2\text{H}_5 & \rightarrow & \text{HC—C}_6\text{H}_{11} & \leftarrow & \text{HC—C}_6\text{H}_5 \\ | & & | & & | \\ \text{CH}_3 & & \text{CH}_3 & & \text{CH}_3 \end{array}$$

II.

and as it is known that all the transformations given in Table I are accomplished without change in configuration, it is possible to follow the effect on rotation of the replacement of the higher aliphatic group by a phenyl group. The effect in the acid itself has already been mentioned. Another striking fact is the influence of the replacement in the nitrile. If the nitriles derived from the dextro-phenylmethylacetic acid are compared with those from the dextro-cyclohexylmethylacetic acids, it is noticed that the replacement of an aliphatic by a phenyl group brings about a change in the direction of rotation. Thus dextro-phenylmethylacetic acid leads to a levo-nitrile, whereas dextro-cyclohexylmethylacetic acid leads to a dextro-nitrile, as should be the case in the dextro-*n*-hexylmethylacetic acid series. The substances derived from the mixed nitriles all rotate in opposite directions from those of the aliphatic series, the only exception being the amine which, in the case of 2,2-phenylmethylethylamine, rotates in the same direction, as should be expected in the purely aliphatic substance.

The phenylethylacetic acid on hydrogenation to the cyclohexylethylacetic acid shows a drop in the value of the rotation, the direction remaining unaltered. Again in this case the replacement of the higher aliphatic radical by a phenyl radical in the nitrile leads to a change of the direction of rotation. This, however, is the only derivative of phenylethylacetic acid in which the direction of rotation is altered. On the other hand, if the numerical values of the rotations are compared, the same order of change is observed as in the methyl series; namely,

$$\text{—COOH} > \text{—CH}_2\text{OH} > \text{—CH}_2\text{X} > \text{—C}\equiv\text{N}$$

The most striking difference in the conduct of the derivatives of the two acids is noted in the reduction of the two nitriles to the corresponding amines. Both nitriles are levorotatory whereas the amines derived from them differ in their rotations; that derived from phenylmethylacetonitrile is levorotatory, the levorotation being enhanced, and that derived from the phenylethylamine is dextrorotatory. It would seem, then, that in the series of derivatives which are being described here the replacement of a methyl group by an ethyl group might change the direction of the rotation of the substance. This is rather an unexpected observation.

The dextro-phenylpropylacetic acid on hydrogenation leads to levo-cyclohexylpropylacetic acid (see Table II). This conduct is rather surprizing and somewhat puzzling. The fact that on hydrogenation the three dextro acids mentioned change their rotation in the same direction, namely towards the left, would warrant the conclusion that they are configurationally related in the sense given in figure (II), and if this assumption is correct, then it follows that levo-cyclohexylacetic acid is configurationally related to dextro-methyl- or ethylcyclohexylacetic acids. This result on the phenylpropylacetic acid again is unexpected and needs fur-

## TABLE I.

*Molecular Rotations of Derivatives of Various Acids* ($[M]_{\mathrm{D}}^{25}$ *in Degrees*).

| Series of: | $-\mathrm{C}(=\mathrm{O})\mathrm{OH}$ | $-\mathrm{C}(=\mathrm{O})\mathrm{Cl}$ | $-\mathrm{C}(=\mathrm{O})\mathrm{SH}$ | $-\mathrm{C}(=\mathrm{O})\mathrm{OC_2H_5}$ | $-\mathrm{C}(=\mathrm{O})\mathrm{NH_2}$ | $-\mathrm{CN}$ | $-\mathrm{CH_2NH_2}$ | $-\mathrm{CH_2NH_2H}_x$ | $-\mathrm{CH_2Cl}$ | $-\mathrm{CH_2OH}$ | $-\mathrm{CH_2SH}$ | $-\mathrm{CH_2SO_3H}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phenylmethylacetic acid.** | | | | | | | | | | | | |
| In ether | +89 5 | +116 2 | +156 4 | +90 8 | | −16 6 | −16 7 | | −0.5 | +22.1 | −14.1 | |
| " absolute alcohol | +82.0 | | | | | −13 9 | −27.6 | | −0 8 | +12 7 | −15.1 | |
| " 75 per cent alcohol | +81.4 | | | | +39.3 | | | | | | | |
| " 50 " " " | +81.2 | | | | | −13 6 | | | | | | |
| Without solvent | +96 8 | | | | | −9.3 | −23 9 | | $\frac{-2\ 5}{\delta}$ | | | |
| In water | | | | | | | | −35.4<br>($x$ = Br) | | | | |
| **Phenylethylacetic acid.** | | | | | | | | | | | | |
| In ether | +132.7 | +163.0 | +218 0 | +139.3 | | −40.1 | +14 5 | | +18.9 | +45.2 | +11.6 | |
| " absolute alcohol | +123 2 | | | | | −38.1 | −11.5 | | +16.8 | +56.5 | +10.5 | |
| " 75 per cent alcohol | +122.0 | | | | +32 4 | | | | | | | |
| " 50 " " " | +121.1 | | | | | | | | | | | |
| Without solvent | +139.4 | | | | | −29.0 | +6 8 | | +17.8 | +28.3 | | |
| In water | | | | | | | | −4.9<br>($x$ = Br) | | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cyclohexylmethylacetic acid. | | | | | | | | | | | | |
| In ether.............. | −19.5 | −13 3 | | | | −18.2 | | | | | | |
| " 75 per cent alcohol. | −17 5 | | | | −15 7 | −20.3 | | | | | | |
| Without solvent....... | | | | | | −19.6 | Racemized. | | | | | |
| Phenylpropylacetic acid. | | | | | | | | | | | | |
| In ether.............. | +27.7 | +40 4 | | | | −11.1 | | | | | | |
| " absolute alcohol.... | +24.2 | | | | | | | | | | | |
| " 75 per cent alcohol. | +23 9 | | | | +7.3 | | −5 2 | | | | | |
| " 50 " " " . | | | | | | | | −3.2<br>($x$ = Cl) | | | | |
| Benzylphenylacetic acid. | | | | | | | | | | | | |
| In ether.............. | +82.8 | +105 3 | | +155.7 | | +2.9 | +57.6 | | +30 0 | +39.4 | +30.3 | |
| " 75 per cent alcohol. | +97.7 | | | +140.4 | +92 8 | −6.2 | +57 1 | +62 3<br>($x$ = Cl) | +28.4 | +37.1 | | |
| " 50 " " " . | | | | | | | | | | | | +2.93 |
| Without solvent....... | | +106 3 | | +162.1 | | | $\frac{+53\ 8}{\delta}$ | | | | | |
| In water.............. | | | | | | | | | | | | +30.8<br>(acid) |
| " " ... .......... | | | | | | | | | | | | +39.7<br>(Ba salt) |

ther substantiation. In the series of phenylpropyl carbinol the transformation could not be carried beyond the amine because of racemization. Attempts at the resolution of the amine were not successful.

The changes in the rotations of the derivatives of benzylphenylacetic acid could not be followed on substances obtained from one and the same starting material due to racemization which accompanied the transformation of the acid amide into the nitrile, the optical activity of the nitrile being so low that further transformation into optically active derivatives was no longer practicable. Fortunately, in this case it was possible to resolve the *d,l*-amine through its tartaric acid salt.

TABLE II.

*Molecular Rotations of the Derivatives of Various Acids* ($[M]_D^{25}$).

| Acid series. | Phenylacetic acids | Corresponding cyclohexylacetic acids |
|---|---|---|
| | *degrees* | *degrees* |
| Phenylmethylacetic | −81 8 | −19.5 |
| Phenylethylacetic | −130 8 | −4 0 |
| Phenylpropylacetic | −29 0 | +4.0 |

The rotation of the derivatives of the acid ending with the nitrile showed the same character of change as in the other two acids. The low dextrorotation of the nitrile in ether is not due to racemization entirely, as is seen from the fact that in 75 per cent of alcohol the rotation of the nitrile is reversed from that of the acid. The *d,l*-amine was resolved into its components by means of tartaric acid and the active forms were not correlated with the corresponding acetic acid. The rotations of the carbinol and of the chloride will be discussed below. Before proceeding with this discussion, however, the fact should be emphasized that in the series of derivatives of phenylmethylacetic acid and of its homologues, *the direction of rotation of substances of the same configuration differs from one homologue to another.* These changes can scarcely be accounted for by the differences in the polarities of the methyl, ethyl, or propyl groups.

*The question of special interest to us, however, is that of the respective rotations of the carbinols and of the halides.* In this respect a striking uniformity is observed in the three series in which the carbinols and the halides can be obtained in an optically active condition. *The changes in rotation on passing from the dextro-carbinol to the halide were towards the left.* In the case of 2,2-phenylmethylethanol and the corresponding halide, there was a reversion of the direction of rotation. In the other two cases the sign of rotation was not reversed, yet the drop in the rotation of the halide should be interpreted in the same sense and not on the basis of racemization for the reason that both substances are formed simultaneously by the action of nitrous acid and of nitrosyl chloride respectively. This change of rotation is analogous to that occurring in the derivatives of disubstituted acetic acids of the aliphatic series.

These observations lend further support to the conclusion reached by Levene and Mikeska regarding the configuration of methyl-, ethyl-, and propylphenyl carbinols. It was then assumed that the dextro-carbinol was configurationally related to the levo-halide. Indeed only on this assumption is the change of rotation on passing from the carbinol to the halide to the left, whereas the change is to the right when the rotations of the methyl- or ethylphenyl carbinols are compared, the latter being of a higher numerical value (2).

In the case of benzylphenyl carbinol the evidence furnished by the present observations is less decisive in view of the uncertainty as to the numerical value of the maximum rotation of the benzylphenylchloromethane.

## CONCLUSIONS.

1. There is a lack of uniformity in the direction of rotation of configurationally related homologous derivatives of substituted phenylacetic acids.
2. All dextrorotatory substituted 2-phenylethanols so far observed on passing to the corresponding halides show a change of rotation towards the left.
3. This observation supports the previous conclusion reached by us in regard to the correlation of the secondary aliphatic aromatic carbinols and of their corresponding halides.

## EXPERIMENTAL.

*Resolution of d,l-Phenylmethylacetic Acid.*—88 gm. of the acid were dissolved in 2700 cc. of hot acetone. 192 gm. of quinine were then added and the solution was allowed to stand overnight at room temperature. The quinine salt separated in crystalline form and amounted to 100 gm. 2 gm. of this salt were decomposed with hydrochloric acid and the rotation was determined on the acid thus obtained.

$$[\alpha]_D^{25} = \frac{-4.44^\circ \times 100}{1 \times 8.112} = -54.7^\circ \text{ (in ether).}$$

After repeated recrystallization from hot acetone, an acid was obtained which showed a rotation of

$$[\alpha]_D^{25} = \frac{-9.13^\circ \times 100}{1 \times 15.304} = -59.7^\circ. \quad [M]_D^{25} = -89.5^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-11.57^\circ \times 100}{1 \times 21.332} = -54.2^\circ. \quad [M]_D^{25} = -81.4^\circ \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = -59.1^\circ. \qquad [M]_D^{25} = -96.8^\circ \text{ (without solvent).}$$

On further recrystallization there was only a slight increase in rotation.

The rotatory power of the acid decreased with ionization. When the above acid was neutralized with 1 equivalent of sodium hydroxide, it showed a rotation of only

$$[\alpha]_D^{25} = \frac{-0.27^\circ \times 100}{1 \times 7.388} = -3.7^\circ. \quad [M]_D^{25} = -6.3^\circ \text{ (in water).}$$

*Levo-Phenylmethylacetyl Chloride.*—259 gm. of the corresponding acid, $[\alpha]_D^{25} = -59.7^\circ$ (in ether) were treated with 414 gm. of thionyl chloride (2 mols). The reaction mixture was allowed to stand overnight at room temperature and was then refluxed on the steam bath for 15 minutes. The unchanged thionyl chloride was removed under reduced pressure. The residue itself was not distilled inasmuch as previous experiments had shown that a slight racemization takes place even on distilling the product under highly reduced pressure. It had a rotation of

$$[\alpha]_D^{25} = \frac{-14.78^\circ \times 100}{1 \times 21.496} = -68.8^\circ. \quad [M]_D^{25} = -116.2^\circ \text{ (in ether).}$$

0.1017 gm. substance required 5.68 cc. 0.1 N $AgNO_3$ (titration).
$C_9H_9OCl$. Calculated. Cl 21.36. Found. Cl 19.82.

*Levo-Phenylmethylacetamide.*—50 gm. of the acid chloride described above, $[\alpha]_D^{25} = -68.8°$ (in ether), were dropped slowly with rapid stirring and thorough cooling into 150 cc. of concentrated alcoholic ammonia. The crude crystalline product was filtered off and the mother liquor concentrated to 30 cc. under reduced pressure. A second crop of crystals was obtained which was combined with the first. The amide was then recrystallized from hot water. Yield, 80 per cent. It melted at 92° and showed the following rotation.

$$[\alpha]_D^{25} = \frac{-2.97° \times 100}{1 \times 11.288} = -26.3°. \quad [M]_D^{25} = -39.2° \text{ (in 75 per cent alcohol).}$$

3.525 mg. substance: 9.410 mg. $CO_2$ and 2.245 mg. $H_2O$.
$C_9H_{11}ON$. Calculated. C 73.15, H 7.45.
Found. " 72.79, " 7.12.

*Dextro-Phenylmethylacetonitrile.*—50 gm. of the corresponding amide, $[\alpha]_D^{25} = -26.3°$ (in 75 per cent alcohol), were thoroughly mixed with 47 gm. (1 mol) of phosphorus pentoxide and distilled under a pressure of about 2 mm. The distillate was dissolved in ether, washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The ether was removed and the residue fractionated under a pressure of 8 mm. The nitrile distilled at 109°. Yield, 78 per cent.

$$[\alpha]_D^{25} = \frac{+2.23° \times 100}{1 \times 17.552} = +12.7°. \quad [M]_D^{25} = +16.6° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+1.82° \times 100}{1 \times 17.420} = +10.5°. \quad [M]_D^{25} = +13.7° \text{ (in 50 per cent alcohol).}$$

$$[\alpha]_D^{25} = +7.0°. \quad [M]_D^{25} = +9.3° \text{ (without solvent).}$$

5.785 mg. substance: 17.440 mg. $CO_2$ and 3.425 mg. $H_2O$.
$C_9H_9N$. Calculated. C 82.44, H 6.87.
Found. " 82.21, " 6.62.

*Dextro-2,2-Phenylmethylethylamine.*—This was prepared by catalytic reduction of dextro-phenylmethylacetonitrile. Glacial acetic acid (300 cc.) dried over a few gm. of phosphorus pentoxide, was distilled directly into the absorption tube. 35 gm. of the nitrile, $[\alpha]_D^{25} = +5.61°$ (in ether), and 2 gm. of a platinum catalyst prepared according to the directions of Adams and Shriner were introduced into the tube by means of a dropping funnel. Care was taken to exclude all moisture.

The reduction was carried out under reduced pressure. After the nitrile and catalyst had been introduced into the absorption tube, before the shaking was begun, the air was withdrawn from the apparatus and replaced by hydrogen. The amount of hydrogen required for reduction of the nitrile to the corresponding primary amine was calculated to be 11,794 cc.

When 8700 cc. of hydrogen had been absorbed, the reaction became very slow and the catalyst was therefore reactivated by withdrawing the hydrogen from the apparatus, refilling it with oxygen, and shaking for 10 minutes. Each time when the reaction ceased the catalyst was again reactivated. After 11,500 cc. of hydrogen had been absorbed, the reaction almost ceased and could not be restimulated by further reactivation of the catalyst.

The solution was filtered and the filtrate concentrated under reduced pressure to about 30 cc. On diluting this solution with much water, a small amount of an oil separated which was extracted with ether. This proved to be unchanged nitrile. The mother liquor was rendered alkaline with sodium hydroxide and the amine was extracted with ether. The extract was washed with a little water and then dried over sodium sulfate. The ether was removed and the residue fractionated.

The first fraction distilled at 90° under a pressure of 12 mm. and the second at 185–186° under a pressure of 9 mm.

After redistillation, the first fraction (19.3 gm.) had the following rotation.

$$[\alpha]_D^{25} = \frac{+0.73^\circ \times 100}{1 \times 14.122} = +5.2^\circ. \quad [M]_D^{25} = +7.0^\circ \text{ (in ether).}$$

4.540 mg. substance: 13.320 mg. $CO_2$ and 3.925 mg. $H_2O$.
$C_9H_{13}N$. Calculated. C 80.00, H 9.63.
Found. " 80.00, " 9.67.

The second fraction proved to be a secondary amine. Total yield, 23.3 gm. (calculated 33 gm.).

In another experiment, 25 gm. of nitrile with $[\alpha]_D^{25} = +12.7^\circ$ (in ether) were reduced in 300 cc. of glacial acetic acid in the presence of 2 gm. of the same platinum catalyst. The first fraction showed a rotation of

$$[\alpha]_{\mathrm{D}}^{25} = \frac{+1.44^\circ \times 100}{1 \times 11.592} = +12.4^\circ. \quad [\mathrm{M}]_{\mathrm{D}}^{25} = +16.8^\circ \text{ (in ether).}$$

$$[\alpha]_{\mathrm{D}}^{25} = +17.7^\circ. \qquad [\mathrm{M}]_{\mathrm{D}}^{25} = +23.9^\circ \text{ (without solvent).}$$

The second fraction, a secondary amine, again amounted to about 20 per cent of the total yield.

Some of the primary amine was converted into the hydrobromide in dry ether. The precipitate was washed with ether and dried. It showed a rotation of

$$[\alpha]_{\mathrm{D}}^{25} = \frac{+0.78^\circ \times 100}{1 \times 4.752} = +16.4^\circ. \quad [\mathrm{M}]_{\mathrm{D}}^{25} = +35.4^\circ \text{ (in water).}$$

0.1000 gm. substance required 4.60 cc. 0.1 N $AgNO_3$ (titration).
0.1000 " " " 4.55 " 0.1 " HCl (Kjeldahl).
$C_9H_{14}NBr$. Calculated. N 6.48, Br 37.03.
Found. " 6.37, " 36.80.

*Dextro-2,2-Phenylmethylethyl Chloride.*—This was prepared by the action of nitrosyl chloride on dextro-2,2-phenylmethylethylamine. 15 gm. of the amine, $[\alpha]_{\mathrm{D}}^{25} = +12.4^\circ$ (in ether), were dissolved in 100 cc. of dry ether, cooled to $-55^\circ$, and then treated with an ethereal solution of nitrosyl chloride until the latter was no longer decolorized. Ice water was then added and the halide extracted with ether. The extract was washed with sodium hydroxide, then with water, and finally dried over sodium sulfate. The residue, obtained on the removal of the ether, weighed 7.6 gm. and showed a rotation of

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-6.32^\circ}{\delta} \text{ (without solvent).}$$

The crude material was submitted to fractional distillation. The first fraction distilled at 55–57° under a pressure of 0.8 mm. and weighed 4.7 gm. Optical rotation was as follows:

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-3.35^\circ}{\delta} \text{ (without solvent).}$$

$$[\alpha]_D^{25} = \frac{-1.12^\circ \times 100}{1 \times 23.592} = -4.75^\circ \text{ (in ether).}$$

The second fraction was not distilled, as previous experiments had shown that it could not be distilled without decomposition. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-1.42^\circ \times 100}{1 \times 11.536} = -12.3^\circ \text{ (in ether).}$$

To remove any carbinol that might have been formed during the reaction, the first fraction was dissolved in 20 cc. of dry pyridine and treated with 5 gm. of phthalic anhydride. The mixture was allowed to stand overnight at room temperature. The next day the solution was acidified with an excess of hydrochloric acid and extracted with ether. The ether extract was washed with dilute sodium hydroxide to remove the phthalic acid and the half ester of the same. The extract was then dried, the ether removed, and the residue distilled under a pressure of 1 mm. It boiled at 52–54°.

$$[\alpha]_D^{25} = \frac{+0.13^\circ \times 100}{1 \times 38.944} = +0.33^\circ. \quad [M]_D^{25} = +0.51^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.21^\circ \times 100}{1 \times 40.960} = +0.51^\circ. \quad [M]_D^{25} = +0.79^\circ \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = \frac{+1.78^\circ}{d}. \qquad [M]_D^{25} = \frac{+2.8^\circ}{d} \text{ (without solvent).}$$

0.1152 gm. substance: 0.1036 gm. AgCl (Carius).
$C_9H_{11}Cl$. Calculated. Cl 23.22. Found. Cl 22.24.

*Levo-2,2-Phenylmethylethanol.*—The crude material obtained in the chlorination of dextro-2,2-phenylmethylethylamine, $[\alpha]_D^{25} = +12.4^\circ$ (in ether), with nitrosyl chloride was treated with phthalic anhydride in pyridine solution, as described in the preceding experiment. The crystallized half ester of the carbinol was then isolated in the usual way. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-2.61^\circ \times 100}{1 \times 15.800} = -16.5^\circ \text{ (in ether).}$$

It was decomposed by boiling for 15 minutes with 3 mols of 30 per cent sodium hydroxide solution. An oil separated which was extracted with ether, washed with water, and then dried over sodium sulfate. The ether was removed and the oily residue distilled under reduced pressure. Only 0.3 gm. of the carbinol was obtained. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-1.79° \times 100}{1 \times 11.008} = -16.3°. \quad [M]_D^{25} = -22.1° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-0.76° \times 100}{1 \times 8.552} = -8.9°. \quad [M]_D^{25} = -12.1° \text{ (in absolute alcohol).}$$

4.325 mg. substance: 12.655 mg. $CO_2$ and 3.355 mg. $H_2O$.

| | | |
|---|---|---|
| $C_9H_{12}O$. | Calculated. | C 79.41, H 8.82. |
| | Found. | " 79.79, " 8.68. |

*Dextro-2,2-Phenylmethylethanthiol.*—2 gm. of dextro-2,2-phenylmethylethyl chloride, $[\alpha]_D^{25} = +0.3°$ (in ether), were treated with 3 equivalents of a 10 per cent solution of alcoholic potassium hydrogen sulfide. The mixture was heated for 24 hours at 100° in a sealed tube. Much water was then added, the mercaptan was extracted with ether, washed with water, and dried over sodium sulfate. It was distilled under a pressure of 1.5 mm. The main part boiled at 70–71°, leaving a small residue in the flask.

The mercaptan showed the following rotation.

$$[\alpha]_D^{25} = \frac{+1.79° \times 100}{1 \times 19.320} = +9.3°. \quad [M]_D^{25} = +14.1° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.31° \times 100}{1 \times 3.128} = +9.9°. \quad [M]_D^{25} = +15.1° \text{ (in absolute alcohol).}$$

0.1216 gm. substance: 0.1761 gm. $BaSO_4$ (Carius).

$C_9H_{12}S$. Calculated. S 21.05. Found. S 19.89.

*Dextro-Phenylmethylthiolacetic Acid.*—14 gm. of the corresponding acid chloride, $[\alpha]_D^{25} = \frac{+11.69° \times 100}{1 \times 26.056} = +44.9°$ (in ether) were added drop by drop with stirring to 18 gm. of potassium hydrogen sulfide in 12 cc. of water. On heating the mixture on the steam bath a lively reaction took place. After all the chloride had been added, the reaction was heated on the steam bath for a few more minutes, then diluted with ice water and acidified. The oil which separated was extracted with ether, the extract washed and then dried over sodium sulfate.

On distillation the acid boiled at 136–138° under a pressure of 3 mm. Yield, 13.8 gm. It showed a rotation of

$$[\alpha]_D^{25} = \frac{+10.03^\circ \times 100}{1 \times 16.272} = +61.6^\circ. \quad [M]_D^{25} = +102.3^\circ \text{ (in ether).}$$

0.1362 gm. substance: 0.1862 gm. $BaSO_4$ (Carius).

$C_9H_{10}OS$. Calculated. S 19.27. Found. S 18.78.

*Resolution of d,l-Phenylethylacetic Acid.*—100 gm. of the acid were dissolved in 1000 cc. of hot 50 per cent alcohol. To this were added 154 gm. of cinchonidine and the solution was allowed to stand overnight at room temperature. The first fraction of the cinchonidine salt which separated in crystalline form weighed 121 gm. 2 gm. of this salt when decomposed with concentrated hydrochloric acid yielded an acid which showed a rotation of

$$[\alpha]_D^{25} = \frac{+4.89^\circ \times 100}{1 \times 16.460} = +29.7^\circ \text{ (in ether).}$$

The salt was recrystallized from 50 per cent alcohol until no further increase of the rotation of the free acid was obtained. It was then decomposed and the acid obtained was distilled. It showed a rotation of

$$[\alpha]_D^{25} = \frac{+3.58^\circ \times 100}{1 \times 4.422} = +81.0^\circ. \quad [M]_D^{25} = +132.9^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+5.67^\circ \times 100}{1 \times 7.624} = +74.4^\circ. \quad [M]_D^{25} = +122.0^\circ \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = +89.94^\circ. \qquad [M]_D^{25} = +139.4^\circ \text{ (without solvent).}$$

The sodium salt had a rotation of

$$\cdot \; {}^{25} \quad \begin{array}{c} +0.86^\circ \times 100 \\ 1 \times 10.452 \end{array} \quad +8.2^\circ. \quad [M]_D^{25} = +15.3^\circ \text{ (in water).}$$

*Dextro-Phenylethylacetic Acid Ethyl Ester.*—31 gm. of the corresponding acid, $[\alpha]_D^{25} = +80.9^\circ$ (in ether), were poured slowly into a cooled mixture of 15 cc. of concentrated sulfuric acid with 100 cc. of absolute alcohol. The mixture was allowed to stand overnight at room temperature. The next day, ice and water were added. The precipitated oil was extracted with ether, and the extract washed with water, aqueous sodium hydroxide solution, and again with water. Then it was dried over sodium sulfate and the ether removed. The residue distilled at 80–85° under a pressure of 3 mm. It showed a rotation of

$$[\alpha]_D^{25} = \frac{+10.71^\circ \times 100}{14.844 \times 1} = +72.2^\circ. \quad [M]_D^{25} = +138.6^\circ \text{ (in ether).}$$

5.460 mg. substance: 14.895 mg. $CO_2$ and 3.905 mg. $H_2O$.
$C_{12}H_{16}O_2$. Calculated. C 74.96, H 8.38.
Found. " 74.39, " 8.00.

*Dextro-Phenylethylacetyl Chloride.*—32 gm. of the corresponding acid, $[\alpha]_D^{25} = +80.9^\circ$ (in ether), were treated with 100 gm. (4 mols) of thionyl chloride. A lively reaction took place without heating. The reaction mixture was allowed to stand overnight at room temperature and was then refluxed on the steam bath for 15 minutes. The unchanged thionyl chloride was distilled off under reduced pressure. The acid chloride itself was not distilled as a slight racemization was found to take place even on distilling the product under highly reduced pressure.

The product showed a rotation of:

$$[\alpha]_D^{25} = \frac{+13.89^\circ \times 100}{1 \times 15.596} = +89.0^\circ. \quad [M]_D^{25} = +162.5^\circ \text{ (in ether).}$$

0.1294 gm. substance: 6.90 cc. 0.1 N $AgNO_3$ (titration).
$C_{10}H_{11}OCl$. Calculated. Cl 19.67. Found. Cl 18.92.

*Dextro-Phenylethylacetamide.*—53.5 gm. of the corresponding chloride, $[\alpha]_D^{25} = +89.1^\circ$ (in ether), were added drop by drop with rapid stirring and thorough cooling to 200 cc. of concentrated alcoholic ammonia. The crude product was filtered off and the mother liquor concentrated under reduced pressure. A second crop of crystals was obtained which was combined with the first and the combined material was recrystallized from 50 per cent alcohol until free from ammonium chloride. Yield, 36 gm.

$$[\alpha]_D^{25} = \frac{+1.65^\circ \times 100}{1 \times 8.308} = +19.9^\circ. \quad [M]_D^{25} = +32.4^\circ \text{ (in 75 per cent alcohol).}$$

0.0989 gm. substance: 0.2656 gm. $CO_2$ and 0.0710 gm. $H_2O$.

$C_{10}H_{13}ON$. Calculated. C 73.61, H 8.03.
Found. " 73.23, " 8.03.

*Levo-Phenylethylacetonitrile.*—32 gm. of the corresponding amide, $[\alpha]_D^{25} = +19.9^\circ$ (in 75 per cent alcohol), were mixed with 28 gm. (1 mol) of phosphorus pentoxide and the mixture distilled under a pressure of 2 mm. The distillate was dissolved in ether, washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The ether was removed and the nitrile distilled under a pressure of 4.1 mm. It boiled at 56–57°. Yield, 20.6 gm.

$$[\alpha]_D^{25} = \frac{-2.56^\circ \times 100}{1 \times 9.26} = -27.6^\circ. \quad [M]_D^{25} = -40.1^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-3.33^\circ \times 100}{1 \times 12.704} = -26.2^\circ. \quad [M]_D^{25} = -38.1^\circ \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = -20.0^\circ. \qquad [M]_D^{25} = -29.0^\circ \text{ (without solvent).}$$

6.045 mg. substance: 18.235 mg. $CO_2$ and 3.770 mg. $H_2O$.

$C_{10}H_{11}N$. Calculated. C 82.76, H 7.58.
Found. " 82.26, " 6.97.

*Dextro-2,2-Phenylethylethylamine.*—This was prepared by catalytic reduction of levo-phenylethylacetonitrile. The reduction was carried out in exactly the same way as the reduction of phenylmethylacetonitrile.

6.5 gm. of the nitrile, $[\alpha]_D^{25} = -27.64^\circ$ (in ether), were dissolved in 50 cc. of glacial acetic acid and 0.5 gm. of a platinum catalyst was added. In this case, the reduction proceeded to completion without reactivation. The amount of hydrogen absorbed was 2182 cc. (calculated 2007 cc.).

3.35 gm. of the primary amine were obtained. It distilled at 74° under a pressure of 2.6 mm. and showed the following rotations.

$$[\alpha]_D^{25} = \frac{+1.88^\circ \times 100}{1 \times 19.306} = +9.7^\circ. \quad [M]_D^{25} = +14.5^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-1.16° \times 100}{1 \times 15.024} = -7.7°. \quad [M]_D^{25} = -11.5° \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = +4.56°. \qquad [M]_D^{25} = +6.8° \text{ (without solvent).}$$

6.135 mg. substance: 18.035 mg. $CO_2$ and 5.515 mg. $H_2O$.
$C_{10}H_{15}N$. Calculated. C 80.53, H 10.06.
Found. " 80.16, " 10.06.

In this case, as in the reduction of the phenylmethylacetonitrile, about 20 per cent of the secondary amine was obtained.

Some of the above primary amine was converted to the hydrobromide in dry ether. The precipitate was washed with ether and dried. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.06° \times 100}{1 \times 2.824} = -2.1°. \quad [M]_D^{25} = -4.9° \text{ (in water).}$$

0.1000 gm. substance required 4.35 cc. $AgNO_3$ (titration).
0.1000 " " " 4.25 " 0.1 N HCl (Kjeldahl).
$C_{10}H_{16}NBr$. Calculated. N 6.08, Br 34.78.
Found. " 5.95, " 34.80.

*Dextro-2,2-Phenylethylethyl Chloride.*—This was prepared by the action of nitrosyl chloride on dextro-2,2-phenylethylethylamine. A solution of 10 gm. of the amine, $[\alpha]_D^{25} = +9.2°$ (in ether), was dissolved in 100 cc. of dry ether, cooled to −55°, and then treated with an ethereal solution of nitrosyl chloride until the latter was no longer decolorized. Ice water was then added and the halide extracted with ether. The extract was washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The residue obtained on removal of the ether weighed 4.3 gm. and showed a rotation of

$$[\alpha]_D^{25} = \frac{+3.00° \times 100}{1 \times 21.248} = +14.1° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+14.4°}{\delta} \text{ (without solvent).}$$

The crude material was distilled under a pressure of 1 mm. The first fraction which boiled at 63–65° and of which 3.2 gm. were obtained, was dextrorotatory, as seen from the following figures.

$$[\alpha]_D^{25} = \frac{+1.95° \times 100}{1 \times 15.848} = +12.3° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+13.08°}{\delta} \text{ (without solvent).}$$

The residue which could not be distilled without decomposition showed a rotation of

$$[\alpha]_D^{25} = \frac{+4.49° \times 100}{1 \times 16.176} = +27.8° \text{ (in ether)}$$

and analyzed as follows:

0.1214 gm. substance: 0.0471 gm. AgCl. Found. Cl 9.59.

To remove any carbinol that might have been formed during the reaction, the distillate was dissolved in 20 cc. of dry pyridine and treated with 3 gm. of phthalic anhydride. The mixture was allowed to stand overnight at room temperature. The next day the solution was acidified with an excess of hydrochloric acid and extracted with ether. The extract was washed with dilute sodium hydroxide in order to remove the phthalic acid and the half ester of the same. The extract was then dried, the ether removed, and the residue distilled under a pressure of 1 mm. It boiled at 62–64°.

$$[\alpha]_D^{25} = \frac{+1.21° \times 100}{1 \times 10.840} = +11.2°. \quad [M]_D^{25} = +18.9° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.85° \times 100}{1 \times 8.568} = +9.9°. \quad [M]_D^{25} = +16.8° \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = +10.6°. \qquad [M]_D^{25} = +17.8° \text{ (without solvent).}$$

0.1120 gm. substance: 0.0842 gm. AgCl (Carius).
$C_{10}H_{13}Cl$. Calculated. Cl 21.30. Found. Cl 18.59.

The analysis of the substance showed it to consist of 87.2 per cent halide and 12.8 per cent by-products. Inasmuch as its rotation is $[\alpha]_D^{25} = +11.2°$ (in ether), it follows that unless the by-product had a rotation of $[\alpha]_D^{25} = +87.2°$ (in ether), it could not account for the entire activity of the substance. Hence, it may be assumed that at least part of the optical activity was due to activity of the chloride.

In another experiment 3 gm. of amine with a rotation of $[\alpha]_D^{25} = +10.10°$ (in ether), which had been received from a previous chlorination experiment, were chlorinated and the resulting halide purified as described in the experiment above. The halide obtained showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.28° \times 100}{1 \times 5.056} = +5.53°. \quad [M]_D^{25} = +9.35° \text{ (in ether)}$$

and analyzed as follows:

0.1083 gm. substance: 0.804 gm. AgCl.
$C_{10}H_{13}Cl$. Calculated. Cl 21.30. Found. Cl 18.36.

The residue showed a rotation of

$$[\alpha]_D^{25} = \frac{+1.62° \times 100}{1 \times 14.072} = +11.5° \text{ (in ether).}$$

In this case, as in the previous one, the residue consisted of the halide with some other impurity as seen from the analysis given below.

0.1252 gm. substance: 0.0304 gm. AgCl. Found. Cl 6.00.

*Dextro-2,2-Phenylethylethanol.*—2 gm. of dextro-2,2-phenylethylethylamine,

$$[\alpha]_D^{25} = \frac{+1.88° \times 100}{1 \times 19.306} = +9.7° \text{ (in ether),}$$

were dissolved in 50 cc. of 95 per cent acetic acid and the solution was cooled in an ice-salt mixture. Nitrous oxide was then passed through the solution until the evolution of nitrogen ceased. Then 33 per cent sodium hydroxide was added until the solution was no longer acid to Congo red. Nitrous oxide was then again passed through the solution until the evolution of nitrogen almost ceased. Much water was then added and the precipitate was extracted with ether. The extract was washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The ether was then removed and the residue distilled under reduced pressure. The alcohol was then purified as the ester of phthalic acid as described for the purification of other alcohols. The pure carbinol boiled at 95–96° under a pressure of 4 mm., and showed a rotation of

$$[\alpha]_D^{25} = \frac{+1.06^\circ \times 100}{1 \times 3.536} = +30.0^\circ. \quad [M]_D^{25} = +45.0^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+1.33^\circ \times 100}{1 \times 3.536} = +37.6^\circ. \quad [M]_D^{25} = +56.4^\circ \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = +18.9^\circ. \qquad [M]_D^{25} = +28.3^\circ \text{ (without solvent).}$$

3.465 mg. substance: 10.195 mg. $CO_2$ and 2.875 mg. $H_2O$.
$C_{10}H_{14}O$. Calculated. C 80.00, H 9.33.
Found. " 80.23, " 9.28.

*Dextro-2,2-Phenylethylethanthiol.*—2 gm. of dextro-2,2-phenylethylethyl chloride, $[\alpha]_D^{25} = +11.2^\circ$ (in ether), were treated with 3 equivalents of a 10 per cent solution of alcoholic potassium hydrogen sulfide. The mixture was heated for 7 hours at 100° in a sealed tube. Much water was then added and the mercaptan was extracted with ether, the extract washed with water, and then dried over anhydrous sodium sulfate.

On distillation, a fraction was obtained which boiled at 81° under a pressure of 1.3 mm. It showed the following rotation.

$$[\alpha]_D^{25} = \frac{+1.77^\circ \times 100}{1 \times 25.240} = +7.0^\circ. \quad [M]_D^{25} = +11.6^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.41^\circ \times 100}{1 \times 6.456} = +6.4^\circ. \quad [M]_D^{25} = +10.5^\circ \text{ (in absolute alcohol).}$$

0.1130 gm. substance: 0.1276 gm. $BaSO_4$ (Carius).
$C_{10}H_{14}S$. Calculated. S 19.27. Found. S 15.51.

The second fraction distilled at 178–180° under a pressure of 1.5 mm. Optical rotation was as follows:

$$[\alpha]_D^{25} = \frac{-1.45^\circ \times 100}{1 \times 4.664} = -31.1^\circ. \quad [M]_D^{25} = -102.6^\circ \text{ (in ether).}$$

This substance may be considered to be the disulfide, as shown from the following analysis.

0.1385 gm. substance: 0.2096 gm. $BaSO_4$ (Carius).
$C_{20}H_{24}S_2$. Calculated. S 19.39. Found. S 20.79.

*2,2-Phenylethylethanesulfonic Acid.*—About 2 gm. of dextro-2,2-phenylethylethyl chloride, $[\alpha]_D^{25} = +5.5°$ (in ether), were treated in a sealed tube with 2 gm. of potassium hydrogen sulfide in alcoholic solution. The mixture was heated for 6 hours at 100°. It was then isolated as described in the preceding experiment. Without distillation the substance showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.61° \times 100}{1 \times 5.388} = -11.3° \text{ (in ether)}$$

and analyzed as follows:

0.1046 gm. substance: 0.1338 gm. $BaSO_4$ (Carius).
Mercaptan $C_{10}H_{14}S$. Calculated. S 19.27.
Disulfide $C_{20}H_{26}S_2$. " " 19.40. Found. S 18.53.

Presumably, the substance consisted of a mixture of the mercaptan and disulfide. By comparison with the previous experiment it may be assumed that this substance corresponds to a mercaptan with a rotation of

$$[\alpha]_D^{25} = +3.5°. \quad [M]_D^{25} = +5.8° \text{ (in ether).}$$

For the sake of conserving the material it was not distilled but was oxidized with barium permanganate in 95 per cent acetone. When the permanganate was no longer decolorized, the manganese dioxide was filtered off and the residue alternately washed with acetone and hot water. The combined filtrates were evaporated to dryness. The residue was washed with ether and then taken up with a little water. On addition of alcohol the salt precipitated as a semisolid mass which crystallized on stirring.

The substance analyzed as follows:

9.805 mg. substance: 1.931 mg. $BaSO_4$ (for Ba).
6.545 " " : 5.940 " " (" S).
Equimolecular mixture of acid and barium salt.
$C_{40}H_{54}O_{12}S_4Ba$. Calculated. S 12.91, Ba 13.85.
Found. " 12.44, " 11.73.

The analysis would seem to indicate that the substance was a molecular mixture of the free acid and the barium salt. Lack of material did not permit further purification.

To determine the rotation of the substance and of the corresponding free acid, we proceeded as follows: 0.1178 gm. of the salt was weighed in a flask and diluted to 2.5 cc. Rotation $[\alpha]_D^{25}$ in a 1 dm. tube was 1.30°. Hence the specific rotation of the substance was

$$[\alpha]_D^{25} = \frac{-1.30^\circ \times 100}{1 \times 4.712} = -27.6^\circ \text{ (in water).}$$

0.5 cc. of concentrated hydrochloric acid was then added to the solution directly in the polariscope tube. The total volume was then 3 cc. and the reading −1.11°. On the assumption that the original substance was an equimolecular mixture of acid and salt, it follows that the above corresponds to 0.1017 gm. of free acid per 3 cc.

Therefore,

$$[\alpha]_D^{25} = \frac{-1.11^\circ \times 100}{1 \times 3.390} = -32.7^\circ \text{ (in water).}$$

As neither the original mercaptan nor the oxidation product was obtained in pure form, the results cannot be regarded as reliable.

*Levo-Phenylethylthiolacetic Acid.*—7.8 gm. of the corresponding acid chloride, $[\alpha]_D^{25} = -23.7^\circ$ (in ether), were added drop by drop to a solution of 10 gm. of potassium hydrogen sulfide in 6 cc. of water. The solution was warmed on the steam bath whereupon a lively reaction took place. In a few minutes the reaction was completed. Ice was then added, the solution was acidified, and the acid extracted with ether, washed with water, and dried over sodium sulfate.

On distillation, a fraction was obtained which boiled at 124–125° under a pressure of 13 mm. Yield, 5.65 gm. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-4.35^\circ \times 100}{1 \times 12.348} = -35.2^\circ. \quad [M]_D^{25} = -63.4^\circ \text{ (in ether).}$$

0.1554 gm. substance: 0.1523 gm. $BaSO_4$ (Carius).

$C_{10}H_{12}OS$. Calculated. S 17.77. Found. S 13.47.

*Catalytic Reduction of Phenylmethylacetic Acid to Cyclohexylmethylacetic Acid.*—2.92 gm. of phenylmethylacetic acid with a rotation of $[\alpha]_D^{25} = +36.1^\circ$ (in ether), were dissolved in 100 cc. of glacial acetic acid. 0.3022 gm. of a platinum catalyst was then added. The reduction was carried out as previously described with an occasional reactivation of the catalyst.

When 1292 cc. of hydrogen were absorbed (calculated 1453 cc.) the reduction was discontinued. The solution was filtered and the filtrate concentrated under reduced pressure until an oily residue remained. This was taken up in ether, washed with water, and dried over sodium sulfate. The residue, after removal of the ether, was distilled under a pressure of 4.7 mm. The acid distilling at 120–121° showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.80° \times 100}{1 \times 10.228} = +7.8°. \quad [M]_D^{25} = +12.2° \text{ (in ether).}$$

On standing at room temperature the acid crystallized whereas the unreduced product remained liquid.

4.260 mg. substance: 10.795 mg. $CO_2$ and 3.930 mg. $H_2O$.

| | | | |
|---|---|---|---|
| $C_9H_{16}O_2$. | Calculated. | C 69.23, | H 10.25. |
| | Found. | " 69.10, | " 10.32. |

In another experiment, starting with an acid of $[\alpha]_D^{25} = -54.6°$ (in ether), a reduced product was obtained showing the following rotation.

$$[\alpha]_D^{25} = \frac{-3.14° \times 100}{1 \times 25.088} = -12.5°. \quad [M]_D^{25} = -19.5° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-2.24° \times 100}{1 \times 19.928} = -11.2°. \quad [M]_D^{25} = -17.5° \text{ (in 75 per cent alcohol).}$$

The sodium salt, dissolved in water, showed the following rotation.

$$[\alpha]_D^{25} = \frac{-0.18° \times 100}{1 \times 9.484} = -1.9°. \quad [M]_D^{25} = -3.3°.$$

*Levo-Cyclohexylmethylacetyl Chloride.*—142 gm. of the corresponding acid, $[\alpha]_D^{25} = -12.5°$ (in ether), were mixed with 104 gm. of thionyl chloride (4 mols). A reaction, much livelier than that observed in the chlorination of the corresponding unreduced acid, took place without heating. The reaction mixture was allowed to stand overnight at room temperature and then refluxed on the steam bath for 15 minutes. The unchanged thionyl chloride was distilled off and the residue fractionated. It boiled at 54° under a pressure of 12 mm. Yield, 85 per cent. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-1.11° \times 100}{1 \times 14.624} = -7.6°. \quad [M]_D^{25} = -13.2° \text{ (in ether).}$$

0.1056 gm. substance required: 6.05 cc. 0.1 N $AgNO_3$ (titration).
$C_9H_{15}OCl$. Calculated. Cl 20.57. Found. Cl 20.33.

*Levo-Cyclohexylmethylacetamide.*—153 gm. of the corresponding acid chloride, $[\alpha]_D^{25} = -7.6°$ (in ether), were dropped slowly with rapid stirring and thorough cooling into 350 cc. of concentrated alcoholic ammonia. The crude product was filtered off and the mother liquor concentrated under reduced pressure. On further concentration of the mother liquor a second crop of crystals was obtained which was added to the first. The combined product was then recrystallized by dissolving it in a minimum amount of hot 99 per cent alcohol and precipitating it with water. Yield, 78 per cent. It was free from ammonium chloride and melted at 138–139°.

The optical rotation of the crude product was

$$[\alpha]_D^{25} = \frac{-0.55° \times 100}{1 \times 4.984} = -11.0°. \quad [M]_D^{25} = -17.1° \text{ (in 75 per cent alcohol).}$$

The optical rotation of the analyzed product was

$$[\alpha]_D^{25} = \frac{-0.45° \times 100}{1 \times 4.568} = -9.9°. \quad [M]_D^{25} = -15.3° \text{ (in 75 per cent alcohol).}$$

2.675 mg. substance: 6.695 mg. $CO_2$ and 2.575 mg. $H_2O$.
$C_9H_{17}ON$. Calculated. C 69.68, H 10.96.
Found. " 69.28, " 10.93.

*Levo-Cyclohexylmethylacetonitrile.*—50 gm. of the corresponding amide, $[\alpha]_D^{25} = -11.0°$ (in 75 per cent alcohol), were mixed with 45 gm. (1 mol) of phosphorus pentoxide and distilled under a pressure of 2 mm. The distillate was dissolved in ether, washed with dilute sodium hydroxide and then with water, and finally dried over sodium sulfate. The ether was removed and the nitrile distilled under a pressure of 3 mm. It boiled at 80°. Yield, 84 per cent. It showed a rotation of

$$[\alpha]^{25} = \frac{-2.72^\circ \times 100}{1 \times 20.504} \quad -13.3^\circ. \quad [M]^{25}_{D} = -18.1^\circ \text{ (in ether).}$$

$$[\alpha]^{25}_{D} = \frac{-2.53^\circ \times 100}{1 \times 17.056} = -14.8^\circ. \quad [M]^{25}_{D} = -20.3^\circ \text{ (in 75 per cent alcohol).}$$

$$[\alpha]^{25}_{D} = -14.3^\circ. \qquad [M]^{25}_{D} = -19.7^\circ \text{ (without solvent).}$$

5.785 mg. substance: 17.440 mg. $CO_2$ and 3.425 mg. $H_2O$.
$C_9H_{15}N$. Calculated. C 78.82, H 10.94.
Found. " 78.70, " 11.49.

*2,2-Cyclohexylmethylethylamine.*—This was prepared by catalytic reduction of levo-cyclohexylmethylacetonitrile. The reduction was carried out exactly as in the case of the phenylmethylacetonitrile. 8.15 gm. of a nitrile, $[\alpha]^{25}_{D} = -14.3^\circ$ (without solvent), were dissolved in 200 cc. of glacial acetic acid. 1.33 gm. of platinum catalyst were added. 2200 cc. of hydrogen were absorbed (calculated 2643 cc.).

The reaction mixture was filtered off from the catalyst and the glacial acetic acid removed under reduced pressure. The residue was diluted with water and extracted with ether to remove unchanged nitrile. The aqueous mother liquor was rendered alkaline with excess sodium hydroxide. It was extracted with ether, the ether washed, and then dried over sodium sulfate.

On distillation the amine boiled at 65–66° under a pressure of 2 mm. and was found to be optically inactive.

4.200 mg. substance: 11.775 mg. $CO_2$ and 5.110 mg. $H_2O$.
0.0352 gm. " : 6.53 cc. N (29°, 756 mm.).
$C_9H_{19}N$. Calculated. C 76.59, H 13.47, N 9.92.
Found. " 76.39, " 13.39, " 10.00.

Attempts have been made to resolve the inactive amine by means of tartaric-, camphorsulfonic-, and bromocamphorsulfonic acids, but without success.

*Dextro-Cyclohexylethylacetic Acid.*—This was prepared by catalytic reduction of phenylethylacetic acid. The procedure employed in the reduction of this acid was in every detail similar to the procedure used in the reduction of phenylmethylacetic acid.

3.0296 gm. of the acid, $[\alpha]_D^{25} = +79.8°$ (in ether), were dissolved in 100 cc. of glacial acetic acid and 0.2984 gm. of platinum catalyst was added. 1571 cc. of hydrogen were absorbed (calculated 1231 cc.). The acid was isolated as in the experiment mentioned above. The distilled product showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.34° \times 100}{1 \times 14.372} = +2.4°. \quad [M]_D^{25} = +4.0° \text{ (in ether).}$$

3.345 mg. substance: 8.650 mg. $CO_2$ and 3.245 mg. $H_2O$.
$C_{10}H_{18}O_2$. Calculated. C 70.58, H 10.58.
Found. " 70.51, " 10.85.

*Dextro-Cyclohexylpropylacetic Acid.*—It was prepared by catalytic reduction of phenylpropylacetic acid. This experiment was also carried out according to the directions given for the reduction of the homologous phenylmethylacetic acid.

2.13 gm. of the acid, $[\alpha]_D^{25} = -16.3°$ (in ether), were dissolved in 100 cc. of glacial acetic acid and 0.2986 gm. of platinum catalyst was added. The total amount of hydrogen absorbed was 996 cc. (calculated 800 cc.). The distilled product showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.22° \times 100}{1 \times 10.092} = +2.2°. \quad [M]_D^{25} = +4.0° \text{ (in ether).}$$

4.015 mg. substance: 10.425 mg. $CO_2$ and 3.895 mg. $H_2O$.
$C_{11}H_{20}O_2$. Calculated. C 71.73, H 10.86.
Found. " 70.80, " 10.85.

*Resolution of d,l-Phenylpropylacetic Acid.*—54 gm. of the acid were dissolved in a hot mixture of 300 cc. of 95 per cent alcohol and 8600 cc. of water. To this solution were then added 98 gm. of cinchonidine, whereupon the solution was allowed to stand for 36 hours at room temperature. The first fraction of the cinchonidine salt separated in crystalline form and amounted to 57 gm. 2 gm. of the salt were decomposed with concentrated hydrochloric acid. The free acid showed a rotation of

$$[\alpha]_D^{25} = \frac{+2.50° \times 100}{1 \times 16.784} = +14.9°. \quad [M]_D^{25} = +26.5° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+2.15° \times 100}{1 \times 16.088} = +13.4°. \quad [M]_D^{25} = +23.8° \text{ (in 75 per cent alcohol).}$$

From the mother liquor an acid was obtained which showed a rotation of

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-1.27^\circ \times 100}{1 \times 9.000} = -14.1^\circ. \quad [\mathrm{M}]_{\mathrm{D}}^{25} = -25.1^\circ \text{ (in ether).}$$

After two more recrystallizations of the first fraction, an acid was obtained with a rotation of $[\alpha]_{\mathrm{D}}^{25} = +33.1^\circ$ (in ether). Further crystallization did not improve the rotation and the yield on this last fraction was only about 10 per cent.

*Resolution of d,l-Benzylphenylacetic Acid.*—The acid was resolved as a quinine salt by repeated recrystallization of the latter from 95 per cent alcohol. A fraction was finally obtained which yielded an acid with a rotation of

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-4.41^\circ \times 100}{1 \times 13.336} = -33.1^\circ. \quad [\mathrm{M}]_{\mathrm{D}}^{25} = -74.7^\circ \text{ (in ether).}$$

On decomposition of the mother liquors an acid was obtained with a rotation of

$$[\alpha]_{\mathrm{D}}^{25} = \frac{+8.06^\circ \times 100}{1 \times 19.672} = +41.0^\circ. \quad [\mathrm{M}]_{\mathrm{D}}^{25} = +92.6^\circ \text{ (in ether).}$$

The above probably does not represent the absolute maximum rotatory power of the acid. For the sake of conserving the material no attempt was made to resolve it further.

The relation between the rotatory power of the acid in alcohol and ether, and of its sodium salt can be seen from the figures given below.

$$\text{For acid} \quad [\alpha]_{\mathrm{D}}^{25} = \frac{-3.41^\circ \times 100}{1 \times 22.208} = -15.4^\circ. \quad [\mathrm{M}]_{\mathrm{D}}^{25} = -34.7^\circ \text{ (in ether).}$$

$$\text{For acid} \quad [\alpha]_{\mathrm{D}}^{25} = \frac{-1.62^\circ \times 100}{1 \times 8.928} = -18.1^\circ. \quad [\mathrm{M}]_{\mathrm{D}}^{25} = -41.0^\circ \text{ (in 60 per cent alcohol).}$$

$$\text{For Na salt } [\alpha]_{\mathrm{D}}^{25} = \frac{-3.75^\circ \times 100}{1 \times 21.833} = -17.2^\circ. \quad [\mathrm{M}]_{\mathrm{D}}^{25} = -38.9^\circ \text{ (in water).}$$

The optical activity of the salt was determined in the presence of an excess of 1 mol of NaOH; that is, 1.9896 gm. of the above acid were treated with 2 mols of standard sodium hydroxide and the solution was diluted to 10 cc. The reading in a 1 dm. tube was −3.75°.

*Dextro-Benzylphenylacetyl Chloride.*—10 gm. of dextro-benzylphenylacetic acid,

$$[\alpha]_D^{25} = \frac{+6.94^\circ \times 100}{1 \times 18.948} = +36.6^\circ. \quad [M]_D^{25} = +82.8^\circ \text{ (in ether)},$$

were treated with 20 gm. of thionyl chloride and allowed to stand at room temperature overnight. The next day the solution was refluxed on the steam bath for 15 minutes whereupon the excess of thionyl chloride was removed under reduced pressure. Without further purification the residue showed a rotation of

$$[\alpha]_D^{25} = \frac{+8.11^\circ \times 100}{1 \times 18.864} = +43.0^\circ. \quad [M]_D^{25} = +105.3^\circ \text{ (in ether)}.$$

On distillation under a pressure as low as 0.2 mm. there was a slight racemization, so no attempt was made to purify the chloride further.

*Dextro-Benzylphenylacetamide.*—10 gm. of dextro-benzylphenylacetyl chloride, described above, ($[\alpha]_D^{25} = +43.0^\circ$), were dropped slowly with thorough cooling and rapid stirring into about 5 mols of concentrated alcoholic ammonia. When all the chloride had been consumed, an equal volume of water was added. The amide precipitated immediately. It was then recrystallized from 50 per cent alcohol until free from ammonium chloride. When pure it melted at 134° and showed a rotation of

$$[\alpha]_D^{25} = \frac{+1.96^\circ \times 100}{1 \times 4.752} = +41.2^\circ. \quad [M]_D^{25} = +92.8^\circ \text{ (in 95 per cent alcohol)}.$$

That some racemization had taken place during the reaction is evident from the fact that in another experiment an acid with a rotation of

$$[\alpha]_D^{25} = \frac{+9.32^\circ \times 100}{1 \times 25.336} = +36.8^\circ. \quad [M]_D^{25} = +83.1^\circ \text{ (in ether)}$$

yielded an amide with a rotation of

$$[\alpha]_D^{25} = \frac{+3.96^\circ \times 100}{1 \times 7.808} = +50.7^\circ. \quad [M]_D^{25} = +114.1^\circ \text{ (in 95 per cent alcohol)}.$$

0.1000 gm. substance: 4.01 cc. 0.1 N HCl (Kjeldahl).
$C_{15}H_{15}ON$. Calculated. N 6.22. Found. N 5.61.

*Levo-Benzylphenylacetonitrile.*—10 gm. of the dextro-benzylphenylacetamide described in the preceding experiment ($[\alpha]_D^{25} = +41.2°$) were mixed with 8 gm. of phosphorus pentoxide. The mixture was heated until molten. The nitrile was poured off, dissolved in ether, washed, and finally dried over anhydrous sodium sulfate. When the ether was removed the residue solidified. Without purification it showed an optical rotation of

$$[\alpha]_D^{25} = \frac{-0.38° \times 100}{1 \times 12.724} = -3.0°. \quad [M]_D^{25} = -6.8° \text{ (in absolute alcohol).}$$

The nitrile was then recrystallized several times from ether from which it crystallized well when cooled to about −55°. About 2 gm. of material were obtained which showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.89° \times 100}{1 \times 11.316} = -7.9°. \quad [M]_D^{25} = -16.3° \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = \frac{+0.69° \times 100}{1 \times 18.616} = +3.7°. \quad [M]_D^{25} = +7.7° \text{ (in ether).}$$

0.1000 gm. substance: 4.60 cc. 0.1 N HCl (Kjeldahl).
$C_{15}H_{13}N$. Calculated. N 6.74. Found. N 6.44.

In the majority of cases the nitrile obtained by this method was either inactive or only slightly active.

*Reduction of Benzylphenylacetonitrile.*—15 gm. of the nitrile were dissolved in 300 cc. of 95 per cent alcohol. 30 gm. of sodium were then added with rapid stirring to the boiling solution. When all the sodium had dissolved, ice and water were added and then an excess of hydrochloric acid. The alcohol was removed under reduced pressure. An oil collected at the surface of the aqueous solution. This was extracted with ether and set aside for later examination. The aqueous mother liquor was made strongly alkaline with sodium hydroxide and the amine extracted with ether, dried over anhydrous sodium sulfate, and finally distilled under a pressure of 0.6 mm. It boiled constant at 128–129°. Yield, 5 gm.

The ethereal extract of the acid solution mentioned above was washed with water and dried over sodium sulfate. The residue crystallized on standing. On recrystallization from petrolic ether a snow-white substance was obtained which melted at 52°. This proved to be 2,3-diphenylpropane.

3.720 mg. substance: 12.550 mg. $CO_2$ and 2.685 mg. $H_2O$.
$C_{14}H_{14}$. Calculated. C 92.37, H 7.69.
Found. " 91.99, " 8.07.

*Resolution of d,l-Benzylphenylethylamine.*—23.5 gm. of the amine were poured into 16.7 gm. of tartaric acid dissolved in 500 cc. of water. The solution was allowed to stand at room temperature overnight. The crystalline precipitate was filtered off and recrystallized once more from 500 cc. of water. The salt was then decomposed with sodium hydroxide and the amine distilled under a pressure of 0.6 mm. The amine showed a rotation of

$$[\alpha]_D^{25} = \frac{+4.40^\circ \times 100}{1 \times 16.264} = +27.1^\circ. \quad [M]_D^{25} = +57.1^\circ \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = \frac{+5.26^\circ \times 100}{1 \times 19.264} = +27.3^\circ. \quad [M]_D^{25} = +57.6^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+31.21^\circ}{\delta}. \qquad [M]_D^{25} = \frac{+65.85^\circ}{\delta} \text{ (without solvent).}$$

In another experiment an amine was obtained with a rotation of

$$[\alpha]_D^{25} = \frac{+9.61^\circ \times 100}{1 \times 23.960} = +40.1^\circ. \quad [M]_D^{25} = +84.6^\circ \text{ (in ether).}$$

Even this rotation could have probably been improved upon by further recrystallization but as in the previous experiments, no such attempt was made.

The levorotatory fraction was obtained from the mother liquors.

The hydrochloride of the amine was easily obtained by saturating an ethereal solution of the amine with dry hydrochloric acid gas. The rotation of the hydrochloride was

$$[\alpha]_D^{25} = \frac{-0.64^\circ \times 100}{1 \times 3.996} = -16.0^\circ. \quad [M]_D^{25} = -39.6^\circ \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = \frac{-0.62^\circ \times 100}{2 \times 3.372} = -18.4^\circ. \quad [M]_D^{25} = -45.6^\circ \text{ (in water).}$$

0.1117 gm. substance: 4.43 cc. 0.1 N $AgNO_3$ (titration).
$C_{15}H_{18}NCl$. Calculated. Cl 14.34. Found. Cl 14.07.

*Dextro-2,2-Benzylphenylethanol.*—2 gm. of dextro-2,2-benzylphenylethylamine, $[\alpha]_D^{25} = +27.1°$, were dissolved in 60 cc. of 10 per cent sulfuric acid and cooled to 0°. Nitrous oxide was then passed through the solution until there was no further evolution of nitrogen. Much water was then added and the carbinol was extracted with ether. The extract was washed first with dilute sodium hydroxide, then with water, and finally dried over ether. The ether was removed and the residue distilled under reduced pressure. It was dextrorotatory as seen from the figures given below.

$$[\alpha]_D^{25} = \frac{+0.76° \times 100}{1 \times 4.088} = +18.6°. \quad [M]_D^{25} = +39.4° \text{ (in ether)}.$$

$$[\alpha]_D^{25} = \frac{+0.68° \times 100}{1 \times 3.882} = +17.5°. \quad [M]_D^{25} = +37.1° \text{ (in 95 per cent alcohol)}.$$

*Dextro-2,2-Benzylphenylethyl Chloride.*—This was prepared by the action of nitrosyl chloride on 2,2-benzylphenylethylamine. 15 gm. of the amine with a rotation of

$$[\alpha]_D^{25} = \frac{+5.26° \times 100}{1 \times 19.264} = +27.3°. \quad [M]_D^{25} = +57.6° \text{ (in ether)}$$

were dissolved in 100 cc. of dry ether. This was cooled to −55° and treated with an ethereal solution of nitrosyl chloride until the latter was no longer decolorized. Water was then added and the halide extracted with ether and finally dried over anhydrous sodium sulfate. When the ether was removed the residue weighed 7.5 gm. The unchanged amine was recovered and again treated with nitrosyl chloride. This was repeated until 13 gm. of the crude material were accumulated. To remove any carbinol that might have been formed during the reaction, the crude material was dissolved in 26 cc. of dry pyridine and treated with 9 gm. of finely pulverized phthalic anhydride. The mixture was allowed to stand at room temperature overnight. The next day the solution was acidified with excess of hydrochloric acid and extracted with ether. The ether extract was washed with dilute sodium hydroxide to remove the phthalic acid and the half ester of the same. The ether extract was then dried, the ether removed, and the residue distilled under a pressure of 0.3 mm. The halide distilled at 130–132°.

$$[\alpha]_D^{25} = \frac{+0.89° \times 100}{1 \times 7.308} = +12.2°. \quad [M]_D^{25} = +28.1° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+1.15° \times 100}{1 \times 9.380} = +12.3°. \quad [M]_D^{25} = +28.3° \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = +9.08°. \qquad [M]_D^{25} = +19.8° \text{ (without solvent).}$$

0.1042 gm. substance: 0.0636 gm. AgCl (Carius).
$C_{15}H_{15}Cl$. Calculated. Cl 15.39. Found. Cl 15.10.

*Levo-2,2-Benzylphenylethanthiol.*—3 gm. of dextro-2,2-benzylphenylethyl chloride,

$$[\alpha]_D^{25} = \frac{+1.19° \times 100}{1 \times 9.160} = +13.0°. \quad [M]_D^{25} = +30.0° \text{ (in ether),}$$

were treated with 3 equivalents of a 10 per cent solution of alcoholic potassium hydrogen sulfide. The mixture was heated for 12 hours in a sealed tube at 100°. Water was then added and the mercaptan was extracted with ether, washed with water, and dried over anhydrous sodium sulfate. It was distilled under a pressure of 0.3 mm. It boiled at 123–127°.

$$[\alpha]_D^{25} = \frac{+1.01° \times 100}{1 \times 7.592} = +13.3°. \quad [M]_D^{25} = +30.3° \text{ (in ether).}$$

0.1406 gm. substance: 0.1090 gm. $BaSO_4$.
$C_{15}H_{16}S$. Calculated. S 14.03. Found. S 10.65.

*Dextro-2,2-Benzylphenylethanesulfonic Acid.*—1.5 gm. of the above 2,2-benzylphenylethanthiol were dissolved in 100 cc. of acetone and 2 cc. of water. This was then treated with an acetone solution of barium permanganate until it was no longer decolorized. The manganese dioxide was then filtered off, and washed alternately with hot water and acetone. The combined filtrates were evaporated to dryness under reduced pressure. The residue of barium salt was washed with ether to remove unchanged mercaptan. It was dissolved in water from which it separated on cooling as a viscous precipitate. This was then stirred several times with dry ether. It solidified on standing overnight. Without any further treatment the salt was pure as seen from the analysis given below. It showed the following rotation.

$$\text{For Ba salt } [\alpha]_D^{25} = \frac{+0.54° \times 100}{1 \times 4.680} = +11.5°. \quad [M]_D^{25} = +39.7° \text{ (in water).}$$

6.970 mg. substance: 2.370 mg. $BaSO_4$ (for Ba).
0.0802 gm. " : 0.0546 gm. " ( " S) (Carius).
$C_{30}H_{50}S_2O_6Ba$. Calculated. Ba 19.98, S 9.35.
Found. " 20.00, " 9.31.

To determine the rotation of the free sulfonic acid, 0.1170 gm. of the barium salt was treated with 0.5 cc. of concentrated hydrochloric acid and diluted to 3 cc. The reading in a 1 dm. tube was +0.35°. Hence,

$$[\alpha]_D^{25} = \frac{+0.35° \times 100}{1 \times 3.132} = +11.2°. \quad [M]_D^{25} = +30.8° \text{ (in water).}$$

The rotation of the free acid was also determined in 50 per cent alcohol. 0.1356 gm. of the barium salt was treated with 0.5 cc. of concentrated hydrochloric acid and 2.5 cc. of absolute alcohol. The solution was then diluted to 5 cc. with water. In a 1 dm. tube the rotation was found to be +0.23°. Hence,

$$[\alpha]_D^{25} = \frac{+0.23° \times 100}{1 \times 2.178} = +10.6°. \quad [M]_D^{25} = +29.3° \text{ (in 50 per cent alcohol).}$$

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# A SIXTY CYCLE CONDUCTIVITY ASSEMBLY FOR BIOLOGICAL FLUIDS.

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For certain clinical and biological measurements of conductivity in which no high degree of precision is required, a method is desirable which takes less time to operate and is more economical in use of material to be tested than the standard Kohlrausch method. To meet this need Christiansen (1) introduced the ionometer and Gram and Cullen (2) after testing its accuracy advocated its use in measuring the conductivity of serum. The ionometer is a direct current spring voltmeter connected in series with a capillary type of conductivity cell. Its scale divisions are in arbitrary units which for any given electrolyte in a given cell may be calibrated against known concentrations of the electrolyte. The following method adapted for a 60 cycle alternating current retains the simplicity of operation of the ionometer, has the advantage of the bridge principle, and is in certain respects more convenient. It consists of a 60 cycle, 110 volt bridge with an A. C. galvanometer as a null-point indicator and a cell of high resistance and small capacity.

The 60 cycle bridge employed is a modified form of the sugar-ash resistance bridge, recently described by Parker (3), which is used to determine the amount of electrolyte in sugar. The bridge has a slide wire scale calibrated in reciprocal megohms. By means of a double throw switch a compensator resistance may be inserted in one of the arms of the bridge to correct, respectively, for changes in cell constant and for deviations in temperature from 20°, so that it is possible to obtain directly readings expressed in terms of specific conductivity at 20°. The compensator resistance in our bridge was modified to correct for changes between 7 and 15

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reciprocal cm. in the cell constant range and for changes in temperature for fluids having a temperature coefficient of 1.9 per cent per degree centigrade, which is approximately the temperature coefficient of 0.1 N NaCl. Furthermore, the temperature compensation was designed to reduce all readings of the specific conductivity to 25° instead of 20°.[1]

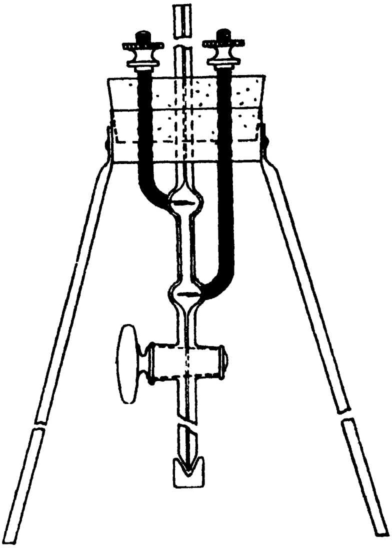

FIG. 1. Pipette cell for use with 60 cycle bridge assembly.

A drawing of the conductivity cell which we designed for use with the assembly is shown in Fig. 1. This cell is of a pipette type of 1 cc. capacity with electrodes 5 mm. in diameter and 25 mm. apart. To avoid polarization effects in a cell of small volume with small electrodes, a high cell constant must be employed. This was obtained by constricting the tube between the electrodes. The constant for the cell shown is about 9 reciprocal cm. From Kohlrausch's data[2] it might be expected that polarization in this

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[1] This bridge was constructed for us through the courtesy of the Leeds and Northrup Company.

[2] Kohlrausch and Holborn ("Leitvermögen der Elektrolyte" Teubner, Leipsic (1916)) obtained satisfactory minima in the telephone with resistances as low as $\frac{15}{n}$ ohms, where $n$ is the area of one electrode in sq. cm.

cell would interfere appreciably in conductivity measurements with solutions giving a resistance below approximately 75 ohms (0.013 mhos). The cell is fitted with a stop-cock and after a measurement is made, the material may be recovered for use in other determinations.

*Comparison of Assemblies and Cells.*—We have made a series of measurements with the ionometer and the 60 cycle bridge and have compared them with a Wheatstone bridge assembly according to the method of Kohlrausch, using a Leeds and Northrup drum-wound slide wire resistance with extension coils, adjustable head phones, and an audiooscillator with a frequency of 1000 cycles. All measuring cells were immersed in a water bath automatically controlled at 25° (± 0.02°). Parker's studies (4) on the calibration of cells would indicate that "the cell having electrodes at the greatest distance gives the most nearly correct value for the specific conductivity of a given solution." Certainly in measurements of higher conductivity where polarization is conspicuous, it may be accepted that the choice of cell is one having the greater distance between electrodes and exhibiting the higher resistance. For this reason we have used with the Kohlrausch assembly a modified type of Henry cell with electrodes approximately 1.5 sq. cm. in area and 10 cm. apart as a standard reference cell. In Table I are given the conductivity measurements of KCl solutions in the Henry and pipette cells with the Kohlrausch and 60 cycle bridge assemblies and in the capillary cell as used with the ionometer. The scale of the ionometer was calibrated against known resistances up to 20,000 ohms; beyond this, the resistance corresponding to the scale readings was calculated from the known resistance of the ionometer.[3]

One of the simplest ways of comparing the accuracy of two or more conductivity methods is to measure a series of solutions by each of the methods and to calculate the ratio of the conductivities obtained for each solution. The so called cell constant ratio obtained should remain constant. The cell constant ratios given

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[3] This calculation was made by using the formula $R_x = \frac{(S_i - S_x)}{S_x} R_i$; where $R_i$ and $R_x$ are the ionometer and unknown resistances and $S_x$ and $S_i$ are the scale readings of the ionometer with and without the unknown resistance in series.

in Table I represent the ratio of the conductivity with the Henry-1000 cycle Kohlrausch system taken as standard to the conductivity of the other cells and assemblies for the same solutions.

TABLE I.

*Conductivity Measurements at 25°.*

| | $\kappa$-Kohlrausch assembly (1000 cycles). | | $\kappa$-60 cycle bridge assembly. | | $\kappa$-Ionometer. | Cell constant ratios. | | | Percentage deviation of 60 cycle assembly from Kohlrausch assembly. | |
|---|---|---|---|---|---|---|---|---|---|---|
| KCl solution. | Henry cell. | Pipette cell. | Henry cell. | Pipette cell. | Capillary cell. | $\frac{\text{Henry-Kohlrausch}}{\text{Pipette-Kohlrausch}}$. | $\frac{\text{Henry-Kohlrausch}}{\text{Pipette-60 cycle}}$. | $\frac{\text{Henry-Kohlrausch}}{\text{Capillary-ionometer}}$. | Henry cell. | Pipette cell. |
| M | *mhos* $\times 10^3$ | *mhos* $\times 10^3$ | *mhos* $\times 10^3$ | *mhos* $\times 10^3$ | *mhos* $\times 10^3$ | | | | | |
| 0.05 | $0.5836^6$ | $0.7432^8$ | 0.575 | 0.721 | 0.01405* | 0.7853 | 0.810 | 41.5 | −1.5 | −3.0 |
| 0.10 | $1.139^7$ | $1.451^3$ | 1.13 | 1.45 | 0.0269* | 0.7853 | 0.786 | 42.3 | −0.9 | 0.0 |
| 0.20 | $2.179^6$ | 2.775 | 2.17 | 2.77 | 0.0516 | 0.7854 | 0.787 | 42.3 | −0.5 | −0.2 |
| 0.40 | 4.170 | $5.315^4$ | 4.17 | 5.30 | 0.0930 | 0.7843 | 0.787 | 44.8 | 0.0 | −0.4 |
| 0.70 | 7.042 | $8.952^2$ | 7.04 | 8.93 | 0.1535 | 0.7866 | 0.789 | 45.9 | 0.0 | −0.2 |
| 1.00 | 9.767 | $12.47^4$ | 9.77 | 12.4 | 0.2049 | 0.7830 | 0.788 | 47.7 | 0.0 | −0.8 |
| 2.00 | $18.48^7$ | $23.30^2$ | 18.50 | 23.0 | 0.3424 | 0.7933 | 0.804 | 54.0 | 0.0 | −1.3 |
| 3.00 | 26.21 | 33.17 | 26.10 | 31.8 | | 0.7902 | 0.824 | | −0.4 | −4.4 |
| 4.00 | 32.64 | 41.50 | 32.70 | 39.9 | | 0.7865 | 0.816 | | +0.3 | −3.9 |

* Resistance measured from scale readings and calculated from formula given in foot-note 3.

The $\frac{\text{Henry-1000 cycle}}{\text{pipette-1000 cycle}}$ ratio is practically constant throughout all concentrations studied; the $\frac{\text{Henry-1000 cycle}}{\text{pipette-60 cycle}}$ ratio appears to rise with concentrations above 1 M KCl; and the $\frac{\text{Henry-1000 cycle}}{\text{capillary-ionometer}}$ ratio rises with the concentration throughout the range studied. In concentrations between 0.1 M and 1.0 M KCl solutions the percentage deviations of the 60 cycle bridge measurements are within 1 per cent of those made with the Kohlrausch method. Somewhat greater deviations occur in the more dilute and more concentrated solutions.

The rise of the cell constant ratio of the ionometer amounts to over 30 per cent between concentrations of potassium chloride of 0.05 and 2.0 N. The magnitude of the polarization effect prevents the use of the ionometer for conductance measurements except for relative measurements where the polarization is largely compensated in the calibration of the instrument. In the ordinary use of the ionometer for measurements of serum conductivity the scale is empirically calibrated by standard solutions before use.

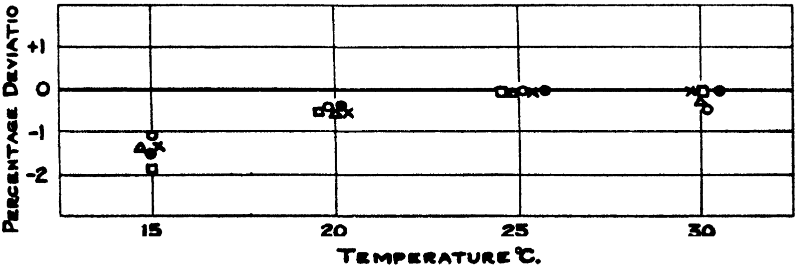

Fig. 2. Percentage deviation from readings of the specific conductivity at 25° when compensator resistance was used to correct for deviation of temperature from 25°. □ represents 0.0551 M NaCl; △, 0.111 M NaCl; ○, 0.199 M NaCl; ×, 0.513 M NaCl; ●, dog serum.

Conductivity measurements of seven pathological sera and two normal urines with the 60 cycle bridge assembly agreed with the Kohlrausch assembly within the limits of −0.6 and +0.5 per cent. These deviations are within the error of the scale readings of the 60 cycle bridge.

*Compensator Resistance for Correction of Temperature.*—The variable resistance which may be used in the 60 cycle assembly to correct the specific conductance to 25° is a convenience in the measurement of fluids whose temperature coefficient is sufficiently close to that of 1.9 per cent per degree centigrade. The filled cell is placed in a large water bath at room temperature and in 2 minutes, after temperature equilibrium is reached, the calibrated compensator resistance is adjusted to correspond to the cell constant and to the temperature of the bath. The specific conductivity is then obtained as if read at 25°. Fig. 2 presents the percentage deviation between readings of salt solution and serum made at various temperatures and corrected by the compensator dial to 25° and the uncorrected readings on the same solutions made at 25°. Measurements between temperatures of 20° and 30° are seen to be within the experimental error.

In recent investigations of the serum electrolytes in pneumonia, Sunderman, Austin, and Camac (5) suggested that with the aid of the Gram and Cullen formula to correct for protein, conductivity measurements might be utilized as an approximate estimate of total base in serum. While this relationship was true in pneumonia patients followed through the course of the disease, further study has indicated that it represents only a special case of the possible relationships existing in serum between conductivity and total base. A study of forty-eight specimens of serum taken from normal subjects and patients with miscellaneous diseases fails to reveal satisfactory correlation between serum conductivity, corrected or uncorrected, and total base.

## SUMMARY.

A conductivity bridge assembly utilizing a 60 cycle lighting current and adapted for measurements of biological fluids has been described. It has been compared with the standard Kohlrausch method and the ionometer.

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# THE METABOLISM OF TRIBUTYRIN.

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The digestibility of the glycerides of the lower fatty acids, their deposition in the fat depots, and their final transformations in the body are matters of considerable uncertainty. Butyric acid, if present in natural fats, exists not as tributyrin but rather as mixed glycerides. If tributyrin could be fed in quantity, its effect on depot fat and on the excretion of acetone bodies would give information of value. But tributyrin is intensely bitter and decidedly toxic. Animals will not willingly eat large amounts of it. Eckstein (1) found that butyric acid could be fed to rats in the form of sodium butyrate up to 19 per cent of the ration. He found the butyryl radical was not deposited in the fat tissues. Our work confirms this.

We have been able to administer tributyrin in three ways: (1) to chickens by pipette, (2) to rats as a part of a mixed ration up to about 9 per cent, and (3) to rats subcutaneously or intraperitoneally.

## *Digestion.*

The digestibility of tributyrin was determined by feeding it to chickens. Rats could not be used because they scattered so much feed that it was impossible to separate it quantitatively from the feces. Any amount of tributyrin could be given chickens by pipette. By using this technique there was no chance of getting any tributyrin in the feces except the undigested portion which was excreted.

The procedure used is as follows: The chickens were kept in metabolism cages which were set over large evaporating dishes. The feces were collected at regular intervals and the feed collected and weighed.

The basal ration was kept before the chickens at all times. This basal ration contained per 75 gm.:

| | gm. |
|---|---|
| Meat scrap (ether-extracted) | 18.0 |
| Starch | 42.5 |
| Cod liver oil | 0.5 |
| Yeast | 10.0 |
| Osborne and Mendel salt mixture (2) | 4.0 |

The amounts of tributyrin given varied with the amount of feed eaten and with the time of the experiment. In general the tributyrin made up about 18 per cent of the feed consumed.

The feces were dried, ground, and extracted with ether. The ether extract was saponified with alcoholic potassium hydroxide. The alcohol was evaporated off and the solution acidified with sulfuric acid, and steam-distilled. The volatile, water-soluble fatty acids were collected, titrated, and calculated as butyric acid. Blank determinations were made on the basal ration. The results given in Table I have been corrected by subtracting this blank. Tributyrin was added to feces and subjected to the same procedure, and it was possible to recover from 97 to 99 per cent of the butyric acid.

Tributyrin was toxic to chickens. Lesions were found in the proventriculus and in the gizzard. The intestinal walls were inflamed and in some cases the mucosa apparently had been destroyed in the fore part of the small intestine. There was some hemorrhage, blood being detected in the feces. In every case the gallbladder was greatly enlarged and often bile had been regurgitated into the gizzard.

Table I gives the results of a set of digestibility determinations. It is seen that tributyrin is digested by chickens. The digestibility falls off if the experiment is repeated with the same chicken. This is probably due to the toxic effect of tributyrin and to damage to the digestive organs.

### *Storage of Tributyrin.*

Rats weighing about 100 gm. were used in these experiments. They were caged individually and weighed every 3 or 4 days. The food was weighed at the same time. The experiments ran about

3 weeks. The diets used in these experiments are given in Table II, and food consumption and body weights in Table III.

The rats were killed by an overdose of chloroform and the depot fat was removed by dissection. This fat together with the hide was extracted with an alcohol-ether mixture which consisted of 3 parts of alcohol to 1 part of ether (3). The alcohol and ether were evaporated off at reduced pressure and the residue extracted with anhydrous ether. This fat was used in the saponification number determinations which were made according to the official method (4). The results of these experiments are given in Table IV.

TABLE I.

*Digestibility of Tributyrin in the Hen.*

| Hen No. | Basal ration fed. | Tributyrin fed. | Tributyrin fed, per cent of ration. | Tributyrin recovered in feces. | Tributyrin digested. | Time of experiment. |
|---|---|---|---|---|---|---|
| | *gm.* | *gm.* | | *gm.* | *per cent* | *hrs.* |
| 1 a | 41.3 | 9.6 | 18.8 | 0.71 | 92.5 | 48 |
| 1 b | 58.5 | 10.2 | 15.0 | 2.7 | 73.5 | 48 |
| 2 | 71.2 | 19.7 | 21.6 | 1.0 | 95.0 | 72 |
| 3 | 46.6 | 31.7* | 40.5 | 3.9 | 87.8 | 48 |
| 4 | 55.0 | 9.9 | 15.2 | 1.5 | 84.9 | 48 |
| 5 a | 43.5 | 10.1 | 19.0 | 0.2 | 98.0 | 48 |
| 5 b | 47.8 | 11.2 | 19 0 | 2.6 | 76.8 | 48 |

* 10 cc. of tributyrin were given every 6 hours.

TABLE II.

*Composition of Diets.*

The quantities of the substances are expressed in gm.

| Feed. | Diet No. | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Tributyrin | 9 | 9 | | 9 | | 9 |
| Lard | 52 | 49 | 61 | | 68 | 58 |
| Starch | 10 | 10 | 10 | 62 | | |
| Casein | 20 | 20 | 20 | 20 | 20 | 20 |
| Yeast | 5 | 5 | 5 | 5 | 8 | 8 |
| Salts* | 4 | 4 | 4 | 4 | 4 | 4 |
| Cod liver oil | | 3 | | | | |
| Ground paper | 2 | 2 | 2 | 2 | 2 | 2 |

* Osborne and Mendel salt mixture (2).

Because of the toxic effect of the tributyrin it was impossible to feed large quantities of it. In order to give rats large quantities of tributyrin it was injected subcutaneously and intraperitoneally. 2 cc. of tributyrin were injected daily for varying lengths of time.

TABLE III.

*Food Consumption and Body Weights of Rats Fed with and without Tributyrin.*

| Rat No. | Initial weight. | Final weight. | Feeding period. | Food consumed. | Diet No. |
|---|---|---|---|---|---|
| | *gm.* | *gm.* | *days* | *gm.* | |
| 1 | 148 | 161 | 21 | 138 | 1 |
| 2 | 116 | 112 | 21 | 111 | 1 |
| 3 | 127 | 149 | 21 | 137 | 1 |
| 4 | 121 | 122 | 21 | 108 | 1 |
| 17 | 192 | 206 | 25 | 172 | 1 |
| 19 | 130 | 158 | 25 | 138 | 1 |
| 7 | 108 | 127 | 21 | 128 | 2 |
| 8 | 131 | 142 | 21 | 131 | 2 |
| 9 | 94 | 109 | 21 | 117 | 2 |
| 11 | 161 | 170 | 25 | 162 | 3 |
| 12 | 186 | 219 | 25 | 216 | 3 |
| 13 | 173 | 195 | 25 | 200 | 3 |
| 14 | 188 | 195 | 25 | 207 | 4 |
| 15 | 185 | 180 | 25 | 212 | 4 |
| 16 | 179 | 195 | 25 | 188 | 4 |

TABLE IV.

*Effect of Tributyrin Feeding on the Composition of Depot Fat in the White Rat.*

| Diet No. | Rat No. | Weight of sample. | KOH (0.5 N) required for saponification. | Saponification No. |
|---|---|---|---|---|
| | | *gm.* | *cc.* | |
| 1 | 1, 2, 3, 4 | 2.542 | 17.6 | 193.5 |
| | | 2.243 | 15.4 | 193.0 |
| 1 | 17, 19 | 2.172 | 15.4 | 198.5 |
| | | 1.844 | 12.8 | 197.0 |
| 2 | 7, 8, 9 | 2.695 | 18.3 | 190.5 |
| | | 1.522 | 10.3 | 191.0 |
| 3 | 11, 12, 13 | 2.891 | 20.2 | 196.0 |
| | | 2.220 | 15.2 | 194.0 |
| 4 | 14, 15, 16 | 2.426 | 17.1 | 197.5 |
| | | 2.620 | 18.2 | 195.0 |

The rats were killed by an overdose of chloroform and before the fat was removed all unabsorbed tributyrin was carefully washed off. The following tabulation gives the saponification numbers which were obtained in these experiments.

| Rat No. | Tributyrin injected. | Saponification No. | Injection. |
|---|---|---|---|
| | *cc.* | | |
| 31 | 4 | 288.8 | Subcutaneous. |
| 32 | 4 | 283.8 | " |
| 33 | 4 | 280.5 | Intraperitoneal. |
| 34 | 4 | 285.6 | " |

10 cc. of tributyrin were injected intraperitoneally into a large vigorous rat. 2 hours later the rat was found dead. On autopsy much of the tributyrin was recovered from the body cavity. The internal organs appeared to be normal. The experiment was repeated and the rat observed continuously after the injection of the tributyrin.

The tributyrin had a decided anesthetic action. In about 15 minutes anesthesia was complete. The rate of breathing became very slow and ceased entirely 1 hour and 12 minutes after the injection of the tributyrin. On autopsy the heart was found to be tightly contracted. The internal organs appeared to be normal. The experiment was repeated with similar results, the rat dying in less than 1½ hours.

The fat of these animals was examined as in previous experiments and the saponification number was the same as that of normal rat fat. Because of the short time between the injection of the tributyrin and the death of the animal one would not expect much fat storage.

### *Effect of Tributyrin on Formation of Acetone Bodies.*

According to the $\beta$-oxidation theory a diabetic animal should excrete more acetone bodies when fed tributyrin than when fed an equal amount of fats having longer fatty acid chains. We tested this by using rats which were rendered diabetic by phlorhizin. Diets 1, 3, 5, and 6, whose composition is given in Table II, were used in these experiments.

The procedure of Levine and Smith (5) was followed with a few modifications. The rats were kept in cages so constructed that the urine could be collected under mineral oil. A solution of sodium fluoride was used as a preservative for the urine. The urine was collected every 2nd day and was immediately analyzed. The phlorhizin was suspended in olive oil and was injected daily. The daily dose was 25 mg. for the first series and 37.5 mg. for the second series of experiments.

The acetone bodies were determined according to the method of Van Slyke (6). β-Hydroxybutyric acid, acetoacetic acid, and acetone were determined together and reported as acetone. Sugar and nitrogen were determined in several samples. Sugar was determined by the Bertrand-Walker-Munson (7) method. A modified Kjeldahl method was used to determine the total nitrogen.

## DISCUSSION.

Tributyrin is digested by the hen but the digestibility falls off if the same hen is used for a second trial. The average digestibility for all determinations was 86.9 per cent. The average of five first trials was 91.6 per cent. It is believed that this decrease in digestibility is due to injury of the digestive tract by the tributyrin.

It is interesting to note that during the steam distillation of hen feces for the determination of butyric acid insoluble volatile fatty acids were obtained along with the soluble volatile acids. The amount of insoluble volatile acids was less in the blank determinations than in the experiments in which tributyrin was fed. This suggests that some synthesis of higher fatty acids had taken place in the intestine.

When tributyrin was injected into rats there was an increase in the saponification number of the depot fat, which indicates that there is some storage of tributyrin. There was not a great deal of difference between results with subcutaneous and intraperitoneal injections; the saponification number was raised to over 280.0 in every case. On the other hand when tributyrin was fed there was no increase in the saponification number of the depot fat. These results indicate that there is no storage of tributyrin when it is fed.

Since tributyrin was digested and absorbed and was not stored in the animal body, what was its fate? There are several possible answers to this question. The butyric acid might have been transformed to higher fatty acids as it passed through the intestinal wall or elsewhere, it might have been oxidized at once and spared other fats to be stored, or thirdly, it might have been converted to glucose. Our experiments lead us to believe that the butyric acid was transformed in part to higher fatty acids. From the fact that the injection of tributyrin increased the saponification number of the depot fat, while the feeding of it did not, it seems probable that the fat is transformed before entering the blood. The presence of increased amounts of insoluble volatile fatty acids in the feces after feeding the tributyrin might be explained on the basis that higher fatty acids are being built up in this transformation. Leube (8) found that the Reichert-Meissl number of the depot fat of a dog did not change when large quantities of butter were fed. Hughes (9) observed that short chain fatty acids were not stored in the animal body. All of this evidence supports the view that the fat is transformed but of course does not describe the mechanism of the change. An increased excretion of acetone bodies following tributyrin feeding with starch-free diets indicates that a part of the absorbed tributyrin was rapidly oxidized. The amount of glucose excreted by phlorhizinized rats fed tributyrin does not indicate a conversion of butyrin to glucose. It was observed that the presence of carbohydrates in a diet high in lard has a marked antiketogenic effect in the phlorhizinized as well as in the normal rat. For example, the removal of the starch from the diet raised the acetone body excretion from 0.57 mg. per 2 day period to 1.6 mg. in the normal, and from 28.8 to 161.0 mg. in the phlorhizinized animal. A probable explanation for the antiketogenic effect of carbohydrate in the phlorhizinized rat is that the phlorhizinized animal is still able to oxidize carbohydrates and when carbohydrates are being oxidized the normal oxidation of fats can occur also. The substitution of tributyrin for lard to the extent of 9 per cent had no marked effect on acetone body excretion provided 10 per cent of starch also was in the food mixture. A rat receiving no tributyrin excreted 0.57 and 28.8 mg. of acetone bodies per 2 day period without and with phlorhizin respectively. A rat that received tributyrin similarly excreted 0.50 and 26.6 mg. When the diet was very low in carbohydrate, containing only small amounts from the yeast and impure casein, but was high in lard, the average excretion of acetone bodies per 2 day period was 2.1 mg. when no phlorhizin was given and 196.9 mg. when phlorhizin was administered. When tributyrin was added to the starch-free diet the acetone body excretion per 2 day period averaged 2.9 and 324.8 mg. without and with phlorhizin respectively.

## SUMMARY.

The average digestibility of tributyrin as determined by seven trials with five hens was 86.9 per cent. If two second trials are excluded the average of the first trials was 91.9 per cent. The toxicity of tributyrin is believed to be the cause of the decrease in digestibility when the same hen is used for the second trial.

There was some storage of tributyrin in the depot fat when it was injected subcutaneously or intraperitoneally. The saponification number of the depot fat from injected rats was about 285, that from control rats was 195.

No tributyrin was stored in the depot fat after tributyrin was fed. It is believed that the fat was transformed in part as it passed through the intestinal wall. The remainder probably was oxidized rapidly.

The amount of acetone bodies excreted by both normal and phlorhizinized rats increased when carbohydrate was removed from the diet. When tributyrin was fed the excretion of acetone bodies increased, provided the diet used was very low in carbohydrate; otherwise tributyrin had no such effect. These facts are in agreement with the theory of $\beta$-oxidation of the fatty acids and the antiketogenic effect of carbohydrate metabolites.

The quantitative relations between carbohydrate oxidation and fat oxidation that fix a maximum ketogenic-antiketogenic ratio for man beyond which acetone bodies appear in the urine in large amounts do not hold at all for the rat.

It is a pleasure to acknowledge indebtedness to Professor J. F. Lyman for valuable advice and criticism.

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# AN IMPROVED DISTILLATION METHOD FOR THE DETERMINATION OF UREA IN BLOOD.

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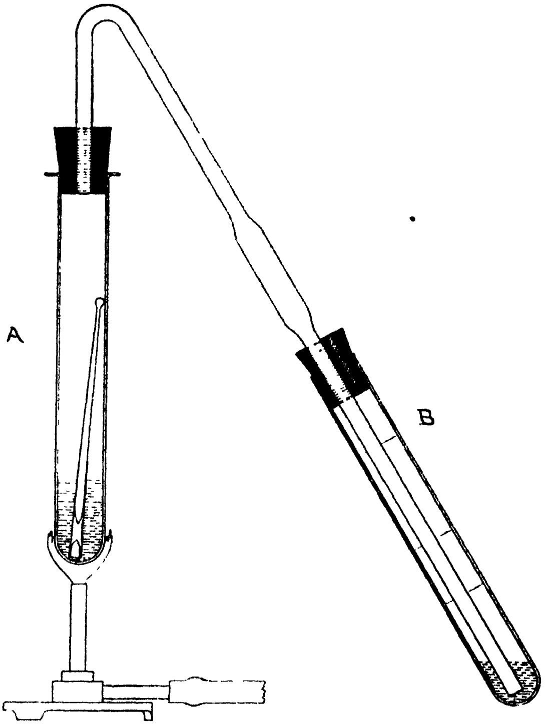
Since the discovery of the high urease content of the jack bean, the enzymatic decomposition of urea by jack bean meal extracts has replaced every other process for the preliminary hydrolysis of the urea. Before the ammonia thus produced can be Nesslerized, it must be separated from the protein materials of the jack bean extract. The reason why it is necessary to isolate the ammonia is not so much the production of turbidity, for that could be obviated; the compelling reason is that protein or amino acid materials have a profound effect both on the quality and on the quantity of color which ammonia gives with Nessler's reagent. All short cut methods for the Nesslerization of urea nitrogen are therefore likely to be grossly erroneous or, at least, unreliable.

In the paper by Folin and Wu on blood analysis, the determination of urea received much attention and several combinations of procedures were described, so as to meet the need of different laboratory conditions. The method based on the isolation of the ammonia, from the decomposed urea, by distillation should have been the most generally useful, but it has not met with much favor. The reasons for this apparent neglect of an intrinsically excellent method are not difficult to find. The bumping, foaming, and back suction encountered while trying to distil off the liberated ammonia must have discouraged many who tried to use the method. As our plan of work called for a great many blood urea determinations, we undertook first of all to subject the distillation method to a thorough, critical revision, and we believe that we have succeeded in completely eliminating the three causes for ruined determinations, bumping, foaming, and back suction.

*1. Prevention of Bumping.*—Bumping is an all too well known phenomenon, particularly when boiling alkaline solutions, and none of the ordinary, most simple preventives, such as the addition of talcum powder, glass beads, pebbles, or metal wire is uniformly effective in preventing it. Students often have trouble with it in their macro-Kjeldahl distillations. Yet a fairly simple and quite effective remedy against bumping has been known in organic chemistry for a very long time, but for some reason has not come into general use in biochemical laboratories. This remedy consists simply of a rather fine glass tube closed at one end and open at the other. When the open end of such a tube rests on the bottom of the flask or test-tube, enough motion is produced at the bottom by the air inside the tube to prevent explosive evolutions of steam. This simplest form of antibumping tube is not serviceable for all purposes, and for quantitative work it is better, indeed sometimes essential, to employ a tube of such a construction that it can not suck up, by the effect of a partial vacuum, a part of the liquid remaining in the flask at the end of a distillation. In this form of antibumping tube, the open space in contact with the bottom of the test-tube is only 2 to 4 mm. in length and may advantageously be at least 2 mm. in diameter. Antibumping tubes of this construction can be used over and over again until accidentally broken. They are so generally useful, yet inexpensive, that they can be used almost as freely as pebbles. Our antibumping tubes are of Pyrex glass and are made for us by Eimer and Amend (see Fig. 1).

These antibumping tubes will unfailingly produce smooth and even boiling provided that too much heat is not applied just when the liquid in the tube begins to boil.

*2. Prevention of Foaming.*—It is practically hopeless to try to boil off ammonia from a test-tube in the presence of urease unless some adequate antifoaming reagent is present. In this laboratory we have always used a heavy black mineral oil as antifoaming reagent both in aeration and distillation methods for the removal of ammonia. Our oil was unfortunately referred to in the Folin-Wu paper as paraffin oil, and this one simple inaccuracy in description has probably been responsible for many failures encountered by others in their urea determinations. Paraffin oil is a definite commercial product which is colorless and has only slight antifoaming properties. The most effective antifoaming oil is the crude viscous product which is used as fuel in oil-burning furnaces. Crude fuel oil is, however, unsatisfactory in one respect; it contains much material which is driven off in solid form by steam. The steam which drives over the ammonia will consequently yield turbid distillates when fuel oil is present in the boiling tube. Theoretically, it should be easy to remedy this defect in fuel oil, for all that is required is to subject a batch of the oil to prolonged treatment with live steam. Practically such a purification process has proved rather bothersome, at least when working with more than small batches. It is therefore rather fortunate that it is not necessary to insist on this purification. The fuel oil is so effective that turbid distillates can be avoided by simply cutting down the amount used



Note anti-bumping tube inside tube A

Fig. 1.

for each distillation. Instead of purifying the oil we therefore simply dilute it.

To 1 volume of crude fuel oil add about 10 volumes of toluene. 2 drops of this mixture will completely prevent the foaming if the boiling process is started slowly. The toluene is added to eliminate the viscosity of the mixture. This toluene will produce a transient turbidity in the distillate which should not be confused with the turbidity discussed above.

*3. Delivery Tube for Ammonia Distillation.*—Through the combined effects of the highly efficient antibumping tube and the antifoaming oil mixture, the process of boiling off the ammonia formed from the decomposed urea is made so smooth that the danger of back suction is almost completely eliminated. Nevertheless, since such back suction means a completely ruined determination, it seems best to provide one further preventive against it. This additional protection is obtained by increasing the capacity of the delivery tube. Too much space is not wanted, either in the boiling tube or in the delivery tube, because it is so much easier to drive every trace of ammonia into the receiver if the total space of the system is small. Instead of making the delivery tube from a straight, slender, glass tube we now make it from a 5 cc. pipette. The added capacity thus secured is ample.

*4. Hydrolysis of Urea by Urease.*—According to Sumner, the jack bean meal now available in the market is not so rich in urease as was the case some years ago. For practical urea determinations that fact is of no consequence, for jack bean extracts are still extraordinarily efficient. Indeed, one of the points to be careful about is to use comparatively weak extracts. For 5 cc. of blood filtrate we use 1 cc. of 2.5 per cent extract. This extract is prepared as follows:

Transfer 0.5 gm. of jack bean meal to a clean 50 cc. flask; add 20 cc. of 30 volume per cent of alcohol. Shake for 10 minutes and filter or centrifuge. This extract should always be prepared on the day it is to be used, because on standing even in an ice box it will develop ammonia and will yield too high results. Even freshly prepared extracts contribute some ammonia so that the found urea nitrogen is about 1 mg. per cent too high. It is because of this extraneous ammonia contributed by the jack bean that one should not use more extract or a stronger extract than is really necessary.

For the past several years blood urea determinations made in this department have almost always been made by the help of urease paper, and urease paper shows less tendency to give too high values. Unless the urea to be determined is extremely small, as in micro determinations, no correction is called for.

The preparation of really dependable urease paper from the jack bean meal now in the market, as Dr. Sumner informed us, is rather more difficult than formerly. The process described below does give a urease paper which is perfectly dependable, if used in the right way.

*Urease Paper.*—Transfer to a clean 200 cc. flask 30 gm. of jack bean meal and 100 cc. of dilute alcohol (30 cc. of 95 per cent alcohol diluted to 100 cc.). Add also 1 cc. of the buffer mixture described below. Stopper tightly and shake vigorously for at least 5 minutes and then shake less hard for about 10 minutes. Filter or centrifuge in 15 cc. tubes, the mouths of which have been covered with tin-foil. The centrifuge process is the more satisfactory, if one has a large centrifuge, so that four, six, or eight tubes can be used. The centrifuging should be continued for half an hour, the filtration for less than 2 hours. It does not pay to try to secure the maximum amount of extract; from 30 gm. of jack bean one will, in any case, get enough urease paper for at least 300 determinations. As soon as the extract is available transfer it to a porcelain dish and at once take it up on strips of rather heavy filter paper, Schleicher and Schüll, No. 597, and hang these up to dry over two threads about 15 cm. apart. While drying, the papers should not be exposed to air currents, for blasts of air seem to destroy the enzyme so long as water is present. As soon as the paper strips are thoroughly dry cut them up into pieces about 1 cm. by 2.5 cm. and preserve in wide mouth bottles.

These urease papers will retain their activity for many months and even for years. For the past year or more we have come to believe that the urease papers did not remain active, but in the course of the present investigation it was discovered that the change which comes with age is a change of solubility rather than loss of activity. The urease becomes fixed in the paper and it is only by shaking the solution several times during the digestion that one secures adequate contact and quantitative hydrolysis of the urea.

*5. Buffer Mixture.*—Mixtures of disodium phosphate and monosodium phosphate are still used as buffers for the hydrolysis of urea by urease but they are not the best. An acidified sodium acetate solution is more effective and is more easily prepared. Dissolve 15 gm. of crystallized sodium acetate in a 100 cc. volumetric flask by the help of 50 to 75 cc. of water. Add 1 cc. of glacial acetic acid (about 99 per cent), dilute to volume, and mix.

*6. Borax Solution and Nessler's Reagent.*—These two reagents are still used as described in the original paper and call for no new discussion. The borax is made up as a 4 per cent solution but on cold days some borax may separate so that the reagent can briefly be referred to as saturated borax solution.

*7. Gum Ghatti Solution.*—Ever since the excellent properties of gum ghatti as a protective colloid were discovered we have used it in all doubtful cases of Nesslerization to secure crystal clear solutions. Gum ghatti is not only more effective than gum arabic, but unlike the latter it is practically free from reducing materials and is perfectly free from ammonia. By producing a perfectly uniform dispersion the gum increases the range of true proportionality, an important point in routine analyses. The use of this gum in connection with the determination of urea of unknown blood samples, where one does not know how much urea nitrogen to expect, is therefore very much to be recommended. The required solution is prepared as follows: Fill a 500 cc. cylinder with water and suspend at the top just below the surface in a wire basket (galvanized iron) 10 gm. of gum ghatti. Leave it overnight, but not for 24 hours, and then remove the basket with undissolved material. A little dirt may get into the solution when the wire basket is disturbed but this soon settles and the clear solution may be used without any further purification.

*8. Urea Determination.*—Transfer 5 cc. of tungstic acid blood filtrate to a Pyrex or Jena test-tube having a capacity of about 30 cc. Add 2 drops of the acetate buffer solution and add either 1 cc. of freshly prepared 2.5 per cent jack bean extract or a piece of urease paper. Insert a cork, and then either immerse the tube in a 700 cc. beaker filled with water having an initial temperature of about 45° or let the tube stand at room temperature. The time allowed for the digestion should be 10 minutes in the warm water or 25 minutes at room temperature. A longer digestion period does no harm. If the urease paper is used it is absolutely essential that the test-tube be shaken during the digestion period.

Cool the tube (if warm), remove the cork, and add (*a*) an antibumping tube, (*b*) 2 drops of the antifoaming oil mixture, and finally 2 cc. of saturated borax solution. Connect at once with the delivery tube and a graduated test-tube receiver. The latter contains 1 cc. of 0.1 N acid and 1 cc. of water. Fasten the boiling tube in a clamp and start the distillation by the help of a small responsive micro burner whose flame can be easily and perfectly regulated. As soon as the contents are nearly boiling, cut the flame down sharply so that the 1st minute of actual boiling is very gentle. Then boil briskly for about 3 minutes and finally boil another minute with the delivery tube not touching the liquid in the receiver. The total boiling period from the first beginning of boiling should be only 4½ to 5 minutes.

Transfer 0.1 mg. of ammonium sulfate nitrogen to another test-tube like the receiver, both graduated at 25 cc., add 1 cc. of gum ghatti solution to each, dilute both to a volume of about 20 cc., and add 2.5 cc. of Nessler's reagent. Mix and make the color comparison.

The standard corresponds to 20 mg. per cent of urea nitrogen, and with this one standard one will be able to get dependable values for urea nitrogen lying anywhere between 8 and 40 mg. per cent. In the case of unusual (nephritic) bloods containing more than 40 mg. per cent of urea nitrogen it is best to repeat the determination with less filtrate plus water.

In our hands, this method gives urea nitrogen values no less consistent and dependable than the total non-protein nitrogen values.

Because of the presence of much undetermined nitrogen in filtrates from laked blood, it has been impossible to estimate the degree of accuracy attained in the urea determinations. The situation is different when extracts from unlaked blood are used for here one can account for practically the whole of the nitrogen in terms of known products. Analytical data illustrating this point will be given in a later paper.

# MICRO METHODS FOR THE DETERMINATION OF NON-PROTEIN NITROGEN, UREA, URIC ACID, AND SUGAR IN UNLAKED BLOOD.

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After we had become convinced of the substantial merits of unlaked blood as a basis for blood analysis (1), it became necessary to supply analytical data giving the normal composition of such blood. This program involved analyses of a large number of bloods and since these standard analyses could incidentally serve as controls, it seemed economical as well as suitable to develop simultaneously certain micro methods for the analysis of unlaked blood. There is a perfectly legitimate place for micro methods in so far as they can be made reasonably accurate. Such methods are not only indispensable for studies on small animals, but they should also prove important to life insurance examiners, and even in regular hospital laboratories they are often useful, because of the limited amount of blood that is sometimes available.

Before describing any of the methods given in this paper it seems best to emphasize one guiding principle which we follow in working with unlaked blood whether by macro or micro methods. Our protein-free extracts are always separated from the mixture of protein precipitate and corpuscle sediment before that mixture has had time to undergo much change in color. The change in color from bright red to dark brown must be accompanied by disintegration of the red corpuscles and one cannot then be sure that some of the corpuscle material has not found its way into the supernatant extract. Some darkening during or after the centrifuging is permissible, but it is always more satisfactory to separate the extract from the sediment before the blood cells have undergone much visible change in color.

### *1. Non-Protein Nitrogen (and Urea).*

The method described here is based on the use of 0.2 cc. of blood. It is doubtless possible with slight modifications to apply the method to 0.1 cc. of blood, but it is not sound practice to use only 0.1 cc. if the larger amount can be obtained; hence it seems better to base the description on 0.2 cc. Those who wish to use the smaller amount will need to be doubly careful about the purity of the chemicals.

The following reagents are needed for the non-protein nitrogen determination.

(*a*) *Sulfate-Tungstate Solution.*—Transfer to a 500 cc. volumetric flask, 10 gm. of c.p. anhydrous sodium sulfate and 15 cc. of 10 per cent sodium tungstate solution. Half fill the flask with distilled water and shake until the sulfate has dissolved. Dilute to volume and mix.

(*b*) *Sulfuric Acid.*—Dilute 12 cc. of $\frac{2}{3}$ N sulfuric acid to 100 cc. and mix.

(*c*) *Mixture of Sulfuric Acid, Phosphoric Acid, and Copper Sulfate.*—Transfer first about 50 cc. of water to a 250 cc. volumetric flask, then introduce in the order named, 15 cc. of 85 per cent phosphoric acid, 10 cc. of concentrated sulfuric acid, and 5 cc. of 5 per cent copper sulfate solution. Cool the mixture and dilute to volume. This acid mixture should be kept in a glass-stoppered flask so as to protect it from contamination. The sulfuric acid is the most likely to contain some nitrogen; we use the Baker and Adamson brand.

(*d*) *Standard Ammonium Sulfate Solution.*—From the regular standard ammonium sulfate solution used for the ordinary non-protein nitrogen method, dilute 1 mg. of ammonia nitrogen to a volume of 100 cc.

(*e*) *Regular Nessler Reagent* (*2*).

*Determination.*—Transfer 4 cc. of the sulfate-tungstate solution to a clean, dry 15 cc. centrifuge tube. With a micro blood pipette, graduated in 0.1 cc. and 0.2 cc., introduce 0.2 cc. of blood, not omitting the rinsing with the solution in the tube. Stir with the pipette and let stand for 15 minutes (longer standing does no harm). At the end of about 15 minutes, add 1 cc. of the dilute sulfuric acid solution. Stir carefully, but rather thoroughly, and centrifuge at a fairly good speed for 5 minutes. Decant the colorless clear supernatant solution and transfer 4 cc. of it to a Pyrex test-tube, graduated (all around) at 25 cc. Add 1 cc. of the sulfuric acid-phosphoric acid mixture, also an antibumping tube, of Pyrex glass. Boil off the water and finish the digestion as in the regular Folin-Wu digestion for non-protein nitrogen. Let cool for about 40 seconds and add 3 to 4 drops of water. Then add about 5 cc. more of water and again apply the flame and boil the mixture for about 1 minute to clean out the antibumping tube. Cool the mixture and dilute to a volume of 18 to 20 cc. Transfer to another graduated test-tube 4 cc. of the standard ammonium sulfate solution and 1 cc. of the strong mixture of sulfuric acid and phosphoric acid. Dilute to about 18 cc. Finally, Nesslerize the solutions at about the same time with 4 cc. of the Nessler reagent. Mix and make the color comparison.

*Calculation.*—The standard, 0.04 mg. of N, corresponds to 25 mg. per cent of non-protein nitrogen, since the actual analysis is made on the extract corresponding to 0.16 cc. of blood $\frac{0.04 \times 100}{0.16} = 25$. $\frac{20}{x}$ times 25 gives, therefore, the non-protein nitrogen in mg. per cent when the standard is set at 20 mm. and $x$ is the reading of the unknown. This calculation is not strictly accurate, because no allowance has been made for the volume of the added blood.

If the Nesslerized unknown is seen by inspection to be much stronger than the standard, Nesslerize immediately another standard containing 0.08 mg. of nitrogen plus 1 cc. of the strong acid mixture, and then wait 15 minutes before making the color comparison.

One step in the process described above calls for some discussion. It is practically impossible to boil off the water for the Kjeldahl digestion unless really effective means are used to prevent the occurrence of explosive bumping. The antibumping tube (see the preceding paper (3), pp. 77, 78) will prevent the explosions, but it is also important properly to regulate the flame from the micro burner, particularly when the mixture is just beginning to boil. Some adequate arrangement for securing perfect control over the gas flame is therefore important. The filtrates from unlaked blood normally contain less organic matter to be destroyed than do filtrates from laked blood. The digestion takes, therefore, very little time after the water has been driven off. Unless much sugar is present the mixture becomes colorless almost as soon as the white fumes begin to appear in the tube and 15 seconds later the flame may be removed. The anti-bumping tubes used in this determination should be longer than the ones used in the urea determination because they must be removed before one dilutes to volume.

From the comparative determination of the non-protein nitrogen of normal blood given in Table I it will be seen that the figures obtained by the micro method tend to be just a little higher than the control figures obtained by the macro method. This slight discrepancy is probably due to the combined effects of two different factors: (1) Any trace of nitrogenous products in the reagents, sulfuric acid, phosphoric acid, and sodium sulfate, introduces a larger error in the micro method. (2) The protein precipitation may be relatively a little less perfect at the higher degree of dilution present in the micro method. The figures in Table I represent averages of two determinations and are given in terms of the nearest mg. per cent.

TABLE I.

*Non-Protein Nitrogen of Unlaked Blood as Determined by Micro and Macro Methods.*

| Sample No. | Non-protein N, mg. per cent | |
|---|---|---|
| | Micro method. | Macro method. |
| 1 | 24 | 21 |
| 2 | 22 | 21 |
| 3 | 23 | 21 |
| 4 | 22 | 21 |
| 5 | 20 | 19 |
| 6 | 23 | 21 |
| 7 | 26 | 26 |
| 8 | 23 | 21 |
| 9 | 20 | 22 |
| 10 | 18 | 17 |
| 11 | 74 | 71 |
| 12 | 227 | 215 |

### *Urea.*

For the micro determination of blood urea one precipitates the corpuscles and the plasma proteins exactly as described above for non-protein nitrogen determinations. The urea is then determined, by the help of urease paper, on 4 cc. of the extract representing 0.16 cc. of blood, according to the directions given in the preceding paper (3).

It is best to use only urease paper, rather than freshly prepared jack bean extracts, for the hydrolysis, in order that the extra ammonia (blank) obtained from the urease may be kept as low and as uniform as possible. The blank in this case will be equivalent to about 1.5 mg. per cent of urea nitrogen.

### *2. Uric Acid.*

As late as 1907, Brugsch and Schittenhelm, and other workers interested in gout, had to use from 150 to 300 cc. of blood in order to secure any quantitative values at all for the uric acid in blood. On the basis of 75 cc. of blood, they could make a quantitative test to which they ascribed considerable significance. In 1913, Folin and Denis obtained fairly consistent quantitative figures for the uric acid by applying the colorimetric method to filtrates representing from 15 to 25 cc. of blood. We now find that the uric acid in blood lends itself rather better than any other waste product to micro chemical determinations. It can be determined with a quite satisfactory degree of precision in 0.2 cc. of unlaked blood.

For this determination 0.2 cc. of blood are treated exactly as for the non-protein nitrogen determination described in the preceding section, up to the point where 4 cc. of the extract have been transferred to a test-tube graduated at 25 cc.

For the rest of the process one follows the directions recently described in this *Journal* (4), except that the standard uric acid solution used for the colorimetric comparison is more dilute—1 mg. of uric acid in 500 cc. of water.

Transfer 1 cc., 2 cc., and 4 cc. of the dilute standard uric acid solution to test-tubes graduated at 25 cc. Dilute the first two solutions to a volume of 4 cc. Then add to each of the standards and to the blood extract 5 cc. of the sodium cyanide-urea solution and, after mixing, add 1 cc. of the uric acid reagent to each of the solutions. Shake and let stand for 4 minutes. Heat the unknown and 1 or 2 of the standards for 2 minutes, cool, dilute to volume, mix, and make the color comparison.

*Calculation.*—The standards are equivalent to 1.25, 2.50, and 5 mg. per cent, respectively, of uric acid. If the standard used for the color comparison is set at 20 mm., then $\frac{20}{x}$ times 1.25, 2.50, or 5 gives the uric acid in mg. per 100 cc. of blood. $x$ is the colorimetric reading of the blood extract.

TABLE II.

*Comparative Uric Acid Determinations on Unlaked Blood by Micro and Macro Methods.*

| Sample No. | Uric acid, mg. per cent. | |
|---|---|---|
| | Micro method. | Macro method. |
| 1 | 2.8 | 2.9 |
| 2 | 2.8 | 3.0 |
| 3 | 2.9 | 3.0 |
| 4 | 3.3 | 3.5 |
| 5 | 3 3 | 3.5 |
| 6 | 3.2 | 3.3 |
| 7 | 2.4 | 2.5 |
| 8 | 2.2 | 2.4 |
| 9 | 3.1 | 3.1 |
| 10 | 3.0 | 3.0 |
| 11 | 5.3 | 5.1 |
| 12 | 5.5 | 5.2 |
| 13 | 7.0 | 6.9 |
| 14 | 12.0 | 12.1 |

It may be remarked that the conditions chosen were selected partly to make the calculation simple. In this connection it should be noted that the determination is actually made on the filtrate corresponding to 0.16. cc. of blood.

Very little experience is required to obtain perfectly dependable values by the micro method. But it must not be forgotten that one is dealing with very minute amounts of uric acid. It is therefore quite essential that the uric acid reagent be completely free from phenol reagent and that the cyanide solution shall be substantially perfect. These two reagents in blank experiments must yield practically no color at all.

From the comparative analyses given in Table II it may be seen that the micro method gives figures which agree with those obtained by the standard macro method. The first ten blood samples represented are from normal persons (1st year medical students), but with reference to this method it may be said that the higher uric acid contents of blood are no less easily determined with accuracy than the lower normal values.

TABLE III.

*Showing That the Colorimetric Ferricyanide Method and the Folin-Wu Copper Method Give the Same Values for Blood Sugar of Macro Blood Filtrates from Unlaked Blood.*

| Sample No. | Blood sugar, mg. per cent. | |
|---|---|---|
| | Folin-Wu copper method. | Ferricyanide method. |
| 1 | 42 | 45 |
| 2 | 82 | 78 |
| 3 | 87 | 85 |
| 4 | 116 | 120 |
| 5 | 155 | 156 |
| 6 | 204 | 200 |
| 7 | 236 | 236 |
| 8 | 250 | 250 |
| 9 | 351 | 341 |

### *3. Sugar.*

In three comparatively recent papers from this laboratory (5) is described a very practical micro method for the determination of sugar in blood. The principle of this method, reduction of ferricyanide and measuring the reduction by the Prussian blue obtained, is particularly suitable for use in a micro method where one is often dealing with very minute amounts of sugar. The method described here represents essentially only a transformation of the colorimetric ferricyanide process described by Folin and Malmros into a corresponding method for use with unlaked blood, but in the course of this transformation it became necessary to introduce certain modifications other than merely to prevent the laking of the blood.

All who have worked with alkaline ferricyanide recognize that this reagent is reduced by a greater variety of substances than are alkaline copper solutions. It is for this reason that one cannot use the ferricyanide for the determination of sugar in normal urine. It is for this reason also that ferricyanide methods will tend to give too high blood sugar values with blood filtrates which represent imperfect removal of the protein materials. This difficulty was encountered when we endeavored to secure blood sugar values by the micro method which should correspond to the very low figures which the revised Folin-Wu method gives on unlaked blood extracts. The ferricyanide method and the copper method give substantially identical results when the two methods are applied to the regular macro extracts prepared from unlaked blood. In Table III are given figures illustrating this point.

Since the copper method and the ferricyanide method give substantially the same figures when the methods are applied to the macro extracts from unlaked blood, it becomes fairly obvious that the higher results sometimes obtained when the ferricyanide method was applied to the extracts from 0.1 cc. of blood must be due to a less complete removal of the protein materials in the micro method. The less perfect removal of the blood proteins in the micro method could be due to the fact that in this method one is working with a tenfold greater dilution of the blood than in the macro method. This interpretation proved correct and virtually solved our problem as shown by the figures of Table IV.

Two different forms of the new micro method are described below.

*Micro Method 1.*—In this process the protein precipitation is made exactly as for non-protein nitrogen or uric acid determinations except that one uses 0.1 cc. of blood instead of 0.2 cc. One advantage of this method is, of course, that one uses the same precipitating reagents as for the other determinations, and the other advantage is that after having once added the blood to the sulfate-tungstate solution one can wait for an hour or more before completing the precipitation by the addition of acid. The greatest practical advantage gained by this method is the fact that one is able, if necessary, to repeat the determination without taking another sample of blood.

Transfer to a clean dry centrifuge tube 4 cc. of the solution containing 15 cc. of sodium tungstate and 10 gm. of sodium sulfate in 500 cc. Introduce by the help of a micro blood pipette 0.1 cc. of blood, stir, and let stand for not less than 15 minutes. Then add 1 cc. of the acid sulfate solution (2 gm. of $Na_2SO_4$ and 12 cc. of $\frac{2}{3}$ N sulfuric acid in 100 cc.). Stir and centrifuge for about 5 minutes.

Transfer 2 cc. of the water-clear extract and 2 cc. of water to a test-tube graduated at 25 cc., and to another similar test-tube add 4 cc. of the standard glucose solution described below. Add to each tube 1 cc. of 0.4 per cent potassium ferricyanide solution and 1 cc. of a solution containing 1.6 per cent sodium carbonate ($Na_2CO_3$) and 0.3 per cent NaCN. Heat for 8 minutes, cool, and add 5 cc. of ferric phosphate-gum ghatti solution. Mix well and after 2 or 3 minutes dilute to volume, mix, and make the color comparison. $\frac{20}{x}$ times 100 gives the sugar value in mg. per cent when the standard solution is set at 20 mm. This value is theoreti-

TABLE IV.

*Showing That with the Colorimetric Ferricyanide Method and 0.1 Cc. of Blood One Obtains the Same Blood Sugar Values as the Folin-Wu Method Gives on Macro Filtrates.*

| Sample No. | Blood sugar, mg. per cent. | |
|---|---|---|
| | Micro method. | Folin-Wu copper method. |
| 1 | 31 | 24 |
| 2 | 71 | 69 |
| 3 | 85 | 81 |
| 4 | 84 | 85 |
| 5 | 95 | 94 |
| 6 | 110 | 111 |
| 7 | 110 | 115 |
| 8 | 121 | 123 |
| 9 | 134 | 135 |
| 10 | 163 | 163 |
| 11 | 173 | 180 |
| 12 | 231 | 236 |
| 13 | 249 | 250 |
| 14 | 267 | 267 |
| 15 | 368 | 368 |

cally 2 per cent below the true value as is explained below (unless the standard sugar solution is made to contain 9.8 mg. per liter). Up to 300 or even 350 mg. per cent of sugar can be determined by the method as described and by the help of a single standard, but where the sugar is definitely above 300 mg. per cent, the determination should be repeated with 1 cc. of the filtrate plus 3 cc. of water.

The sugar standard for use with this method should contain per liter (*a*) 10 gm. of anhydrous sodium sulfate, (*b*) 250 mg. of benzoic acid, and (*c*) 10 mg. of glucose, or preferably 9.8 mg. of glucose.

The other required reagents are: (*a*) 0.4 per cent potassium ferricyanide solution; (*b*) a solution containing 1.6 per cent anhydrous sodium carbonate and 0.3 per cent sodium cyanide; (*c*) a solution containing about 20 gm. of gum ghatti, 5 gm. of anhydrous ferric sulfate, and 75 cc. of 85 per cent phosphoric acid per liter. The preparation of each of these reagents is described in the three papers referred to above

*Micro Method 2.*—In connection with sugar determinations in unlaked blood it is indispensable to meet the possible objection that the corpuscle sugar has not been given adequate time for diffusion and the establishment of equilibrium between the cells and the surrounding solution. This condition is provided for both in the regular macro method for preparing unlaked blood extracts and also in micro Method 1, by not adding the acid until after an adequate waiting period. Because of this theoretically important point it seemed at first impractical to use only one solution for the precipitation of the protein as was done in the original micro method. It has been found, however, that by increasing the protecting salt concentration it is possible to use one solution with which the blood may be left for several hours without incurring any visible destruction of the corpuscles. In this method we have still further cut down the dilution of the blood.

The two special solutions needed for this determination are: (*a*) a solution containing 20 gm. of neutral anhydrous sodium sulfate, 10 cc. of 10 per cent sodium tungstate, and 10 cc. of $\frac{2}{3}$ N sulfuric acid in 250 cc.; (*b*) standard glucose solution containing per liter 250 mg. of benzoic acid, 20 gm. of neutral anhydrous sodium sulfate, and 10 mg. of glucose, or preferably 9.5 mg. of glucose.

*Determination.*—With a 2 cc. Ostwald pipette introduce 2 cc. of the acid-sulfate-tungstate solution into a clean dry centrifuge tube, and with a micro blood pipette introduce 0.1 cc. of blood. After rinsing the pipette with the solution and stirring carefully *stopper the tube with a clean cork* and let stand from 15 minutes to 4 hours. Then centrifuge for 5 minutes, without removing the cork. With an accurate 1 cc. Ostwald pipette transfer 1 cc. of the extract to a test-tube graduated at 25 cc. Add also 4 cc. of water. Transfer to another similar test-tube 5 cc. of the standard glucose solution. Then add the other reagents and finish the determination exactly as described under Method 1.

A few explanatory remarks may be added.

1. It will be noted that in Method 2, 5 cc. of the sugar standard are used, and only 4 cc. in Method 1. This is done in order to simplify the calculation so that in each case $\frac{20}{x}$ times 100 gives the sugar in mg. per cent, except for the correction needed, because the final volumes of the diluted blood are 5.1 and 2.1 cc. respectively. This correction is 2 per cent in Method 1, and 5 per cent in Method 2. It is in our opinion safer and better to introduce these corrections than to measure out liquids involving fractions of a cc. in the actual work; one cannot measure 1.9 cc. with the same ease and certainty that one can measure 2 cc. with an Ostwald pipette. An even more practical method for securing simplicity both in the experimental work and in the calculation would be to cut down the sugar contents of the standard sugar solutions by 2 per cent and 5 per cent respectively, so that one would have 9.8 mg. of glucose per liter for the standard of Method 1, and 9.5 mg. per liter for the glucose standard of Method 2.

2. It seems best to mention specifically that in these revised micro determinations we recommend the use of only 1 cc. of the 0.4 per cent potassium ferricyanide solution instead of 2 cc. The reason for this change is that the autoreduction in blank experiments with ferricyanide is materially greater in the presence of much sodium sulfate, and under these conditions is much greater with 2 cc. than with 1 cc. of the ferricyanide.

3. The sodium picrate light filter described by Folin and Malmros should always be used for the colorimetric comparisons and after a time, usually several months, the used picrate paper should

be replaced by a new one. The so called Daylite glass screens are not needed or even desirable in this color comparison, because they cut out too much light.

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# METABOLISM OF THE LUNG-FISH, PROTOPTERUS ÆTHIOPICUS.

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The estivating and air-breathing habits of the Dipnoi, or lung-fishes, have been known to biologists for many years, but no detailed physiological or biochemical observations on them have ever been recorded. Through the favor of the John Simon Guggenheim Memorial Foundation, it was possible for Dr. James P. Baker and the author to collect a number of specimens of *Protopterus æthiopicus,* Heckel from Lake Victoria, Africa, and to bring them alive to New York. A number of these fish have been induced to enter into estivation in the laboratory. The work to be reported here consists of observations on the metabolism of these and active fish, made under controlled conditions.

The biology of the Dipnoi and the collection of the specimens which we have examined have been described elsewhere (Smith, 1930, *b*). The Dipnoi are an archaic group of fishes, characterized by the possession of a lung opening off the ventral side of the esophagus. In *Protopterus æthiopicus* the gills are reduced and inadequate for respiration, and the animal is entirely dependent upon aerial respiration. Though unable to move about to any great extent on land, these fish can live almost indefinitely out of water, and when stranded in desiccating mud-flats they bury themselves beneath the surface, leaving a burrow through which they obtain air. The body becomes invested in a cocoon formed of dried slime and, rigidly imprisoned in the hard earth, the fish pass into a state of estivation which persists until water again covers the land.

A number of lung-fish were induced to estivate artificially by placing them in soft mud which was subsequently allowed to dry out at room temperature and humidity. When thoroughly dry the mud was very hard, and in order to remove the fish the mud had to be chipped away with sharp steel instruments. By careful excavation the fish could be removed in most instances without injury and with little sensory stimulation. The estivating fish is found curled up near the bottom of the mud block, firmly embedded in the mud except where the head protrudes into the more or less perpendicular burrow. The entire fish is closely enveloped at all points by a dry, brownish, parchment-like membrane which is contiguous to the skin over all exposed areas. At the mouth the membrane extends in the form of a short tube between the lips. It is through this oral aperture that the fish obtains air.

The cocoon is tough, insoluble in water, and non-hygroscopic; it constitutes an effective barrier against infection and against loss of water from the body surface. During the removal of the hard mud, parts of the cocoon are necessarily torn away from the fish. The skin beneath is invariably moist (wet is perhaps more accurate) and somewhat slimy, but impressively clean and healthy. The skin of a frog freshly removed from water presents a close analogy. Within a short time the skin dries, showing that the insulation of the cocoon, and not any hygroscopic property of the skin or its secretions, is responsible for its moist condition.

The burrow, descending from the top of the jar, is of nearly uniform diameter (about the diameter of the fish) down to the nest. The latter is entirely filled by the body of the fish, with no free space visible at any point. So closely is the animal encased by the mud that one wonders where there is room for the alternate expansion and contraction of the body incident to respiration. Except for this slight movement, which probably is effected largely in the median line of the curled body, the fish is as effectively immobilized as though it had been covered with concrete. This muscular immobilization affords one of the most interesting aspects of the metabolism studies to be reported later on.

The ureters in *Protopterus* open into the cloaca, which is variably situated on the right or left side of the mid-line somewhat posterior to the origin of the anal fin. In the estivating fish the cocoon fits tightly against the cloaca and effectively closes it. That portion of the cocoon immediately opposite the cloaca is usually stained, the stain sometimes penetrating the cocoon and showing on the adjacent mud wall. This stain is apparently produced by the last urine to be excreted during the early days of estivation. There is never any accumulation of free urine or solid excreta in the cloaca or the cocoon. The fish is entirely deprived of exogenous water after the mud has dried, and from this time on only water arising from the metabolism of foodstuffs and the degradation of tissue can go to form urine. Throughout the period of estivation water must be lost in considerable quantities by way of the respiratory tract and, perhaps, by a low grade evaporation from the moist skin through the cocoon. The general conditions are such as to indicate that urine formation must be suspended early in estivation, shortly after the last free water has disappeared. This condition was reached in our estivating fish about November 1, 1928.

During estivation the fish is in a state of "sleep" or inhibition from which it is not wakened by mild sensory stimulation. In most of our experiments the estivating fish were removed from the mud and placed in the metabolism chamber to measure the gaseous exchange. Since the chamber contained NaOH solution to absorb $CO_2$, the air in the chamber was always saturated with water vapor and this circumstance prevented the fish from drying, even when large areas of the cocoon were torn off. Several fish have been kept in the metabolism chamber in the estivating state for over a month. During this time they showed no movements, nor any change in condition except loss of weight. In other instances the period of estivation has been terminated earlier for experimental purposes by placing the fish in water. The principal difficulty in keeping the fish in estivation after removal from mud is infection, which starts under the cocoon. The development of infection has been the only factor, apart from immersion in water, which in our experience wakens the fish. In awakening due to infection the fish shows increased reactivity to prodding, increased respiratory activity, and increased $O_2$ consumption. In one instance an infected fish broke apart the remnants of the cocoon and wriggled itself out of its curled position. The spontaneous activity was still far below normal, however.

When the estivating fish is placed in water it is wakened in the course of a few hours by the development of asphyxia, for it is of course unable to breathe under water. It ultimately breaks out of the cocoon and struggles to the surface to get air.

Fish recently removed from estivation are particularly susceptible to infection, which ultimately proves fatal. The nature of our experiments has been such that it has been necessary to handle the fish every day in order to replace the water and alkali in the metabolism chamber, etc. Every precaution has been taken to guard against infection, but with the necessary experimental work in progress, most of the fish have died from this cause within 2 to 3 weeks after removal from mud.

A series of analyses of the body fluids of normal lung-fish was made in Africa. Since these agree in all important details, the data on one fish only will be given here (Table I). These data are noteworthy only as a basis for comparison with fish which have been in estivation. Analyses of the muscles of active normal fish are given in Table III, to which subsequent reference will be made.

### *Metabolism of Active Fish.*

Particular interest attaches to the metabolism of *Protopterus* in view of the prolonged estivation which this animal normally undertakes. We have followed the metabolism of two active fish (Nos. 217 and 41) which had never been in estivation. The important data on these fish are summarized in Table II. Throughout the period of observation these fish were kept without food, and during most of this time they were confined to the metabolism chamber in 300 to 500 cc. of water. Our experience has been that after a few hours in the chamber the fish becomes accustomed to its quarters and lies quietly on the bottom, moving only to breathe. The bodily effort involved in this act is slight, since at most the fish need only flex the body so that the head can be lifted momentarily to the surface of the water while the lungs are emptied and filled with air.

The important conclusions to be drawn from these observations concern the metabolic rate, protein combustion, and the excretion of urea and ammonia.

Fish 217 had been feeding for 3 months prior to observation. The condensed data of Table II give the $O_2$ consumption, etc., of this fish over a period of about 10 weeks. Beginning with the 1st day of fasting, the $O_2$ consumption fell to a steady level of 20 cc. per kilo per hour at 20°, which was maintained with fair constancy over a period of 8 weeks. Subsequently this fish was placed in an incubator at 30° to observe the effect of temperature. The $O_2$ consumption rose to a maximum on the 2nd day (41.2 cc. per kilo per hour), after which it declined gradually to a level only one-third above the previous level at 20° (*i.e.*, about 27 cc. per kilo per hour). After about 3 weeks at 30°, the temperature was raised to 40°, but a failure of the thermoregulator led to an overheating of the incubator and the fish was killed.

TABLE I.

*Composition of Body Fluids of Normal Protopterus æthiopicus, ♂.*

| | Plasma. | Perivisceral fluid. | Pericardial fluid. | Urine. |
|---|---|---|---|---|
| B, *mM per liter* | 99.0 | 91.0 | 97.0 | |
| K " " " | 8.2† | 4.2 | 3.2 | |
| Ca " " " | 2.1 | 2.1 | 1.9 | |
| Mg " " " | Trace. | Trace. | Trace. | |
| Cl " " " | 44.1 | 46.0 | 34.0 | Trace. |
| $SO_4$ " " " | Trace. | | | None. |
| $PO_4$ " " " | 1.0 | 1.6 | | |
| $CO_2$ " " " | 35.0‡ | 36.0‡ | | |
| Total N, *mg. per cent* | 900.0 | 91.9* | 68.9* | 36.6 |
| " non-protein N " " " | 14.3 | | | |
| Urea N " " " | 1.7 | | | 6.8 |
| Uric acid N " " " | Trace. | | | 0.3 |
| $NH_3$-N " " " | <1.0 | <1.0 | | 15.1 |
| Creatine N " " " | None. | | | 0.2 |
| Creatinine " " " " | 1.2 | 2.5 | | 2.3 |
| Δ, °C. | 0.396 | 0.410 | 0.351 | |
| " Fish 2, " | 0.480 | 0.415 | 0.418 | |

* High nitrogen may have been due to cells which could not be centrifuged out.

† Slight hemolysis.

‡ By hot titration with bromocresol purple.

Fish 41 when first observed had been fasted about 22 weeks. This fish was kept under fairly continuous observation of one kind or another for a period of nearly 5 months. During this time the $O_2$ consumption rose from 18.1 cc. to 34.1 cc. per kilo per hour. This increase in metabolism was clearly associated with an increase in protein combustion. Such an increase in metabolism has long been recognized as a terminal feature of starvation in mammals. (This fish died, probably from asphyxia, during observations on the effects of low $O_2$ tensions on the $O_2$ consumption.)

During most of the period when Fish 217 and 41 were studied the total nitrogen, urea, and $NH_3$ were routinely determined and afford a basis for estimating the protein fraction in the fuel used.

TABLE II.

*Metabolic Observations on Active Protopterus.*

| | Temperature. | $O_2$ | R.Q. | Urea + $NH_3$-N. | $NH_3$-N | Protein $O_2$. | No. of observations. |
|---|---|---|---|---|---|---|---|
| | *°C.* | *cc. per kg. per hr.* | | *mg. per kg per day* | *mg. per kg per day* | *per cent* | |
| Fish 217; had been feeding for 3 mos ; weight 220 gm. | | | | | | | |
| Fasting. 1st day..... ..... | 26 | 53.3 | | 37.4 | 27.6 | 17.4 | 1 |
| 2nd " .. . . | 20 | 33.8 | | 46.8 | 38.6 | 33.0 | 1 |
| 4th–12th days..... | 20 | 18.6 | | 35.5 | 28.6 | 47.3 | 8 |
| 54th–58th " ..... | 20 | 20.4 | 0.801 | 42.2 | 20.4 | 51.2 | 4 |
| Moved to 30° to observe effect of temperature. | | | | | | | |
| 1st day. . ..... . | 30 | 36.4 | 0.904 | 90.1 | 47.1 | 61.5 | 1 |
| 2nd " . . . .. | 30 | 41.2 | 0.836 | 94.7 | 26.4 | 56.9 | 1 |
| 5th " . .. . . . | 30 | 30.6 | 0.863 | 84.3 | 29.8 | 68.0 | 1 |
| 11th " ......... | 30 | 30.0 | 0.845 | 63.3 | 28.8 | 52.2 | 1 |
| 14th " . . . ..... .. | 30 | 25.7 | 0.965 | 56.0 | 24.2 | 53.8 | 1 |
| 19th " ...................... | 30 | 28.9 | 0.943 | 65.8 | 30.2 | 56.5 | 1 |
| Fish 41; had been fasted but active for 5 mos.; weight 41 gm. | | | | | | | |
| Fasting. 22nd wk................... | 20 | 18.1 | | 47.6 | 33.0 | 65.0 | 5 |
| 27th–30th wk ..... | 20 | | | 72.0 | 48.0 | | 20 |
| 31st–32nd " ....... | 21 | 30.5 | 0.843 | 110.0 | 70.7 | 89.3 | 4 |
| 34th–35th " . ....... | 22 | 34.1 | 0.841 | 118.0 | 92.0 | 85.5 | 9 |
| 42nd wk............... | 20 | | | | | 102.0 | 10 |

It has been shown in the fresh water carp and goldfish that urea and ammonia are excreted by the gills (Smith, 1929). Since the urine obtained from freshly caught and well fed specimens of *Protopterus* contains only traces of these substances (Table I) and otherwise resembles in its composition the urine of fresh water fishes generally, we presume that urea and ammonia are excreted principally by the gills in this fish.

The continuous collection of urine by retention catheter is impossible in fish under prolonged observation, so we have followed the nitrogen metabolism by analyzing the water in which the fish was kept. The fish secretes a small amount of slime, presumably a mucoprotein, which adds to the total N. This moiety does not appear to be significant so far as protein combustion is concerned, for the urea + $NH_3$-N comprises nearly all the total N produced by the degradation of protein in estivating fish, and consequently we have chosen these fractions as a quantitative index of protein metabolism.

With reference again to Fish 217, the combustion of protein (calculated on the basis of the urea + $NH_3$-N and estimating that 1 gm. of N = 5.94 liters of $O_2$) accounts for about 50 per cent of the $O_2$ consumption. It is significant that this protein fraction was reached on the 4th day of fasting, and that it was maintained for several months. On removal to 30°, the protein fraction rose on 1 day to 68 per cent, but subsequently fell again to 52 to 56 per cent.

In the case of Fish 41, the protein fraction was 65 per cent when the fish was first observed, a fact in part attributable to the longer period of fasting. During the next 5 months the protein fraction in this fish rose until at the time of its death during observation, it constituted 100 per cent of the total metabolism. It has already been remarked that coincident with this rise in protein metabolism, the total metabolism increased nearly 100 per cent, indicating abnormal destruction of body tissue.

That protein normally furnishes about 50 per cent of the energy in fasting fish is shown by the observations of Knauthe (1897, 1898), Lindstedt (1914), and Rubner (1924), and we have found a similar fraction of protein energy in fasted frogs (unpublished observations). It was somewhat of a surprize to us, nevertheless, to observe this high ratio in the lung-fish because we expected, in view of its estivating habits, to find an extensive utilization of fat with marked economy of protein. No direct information is available on the relative combustion of fat and carbohydrate, but in fish in general the carbohydrate is quickly depleted (*cf.* Knauthe and Lindstedt). The abrupt rise in protein combustion in Fish

217 when first fasted is indirect evidence of the rapid depletion of the carbohydrate stores.

Concerning the R.Q., we have every confidence in the technical determination of the $O_2$ consumption and $CO_2$ production as carried out here, and checked the methods by the combustion of alcohol in the chamber. But we attach little significance to this ratio physiologically for the reason that we have found the most extreme variations in it. This is probably due to the fact that the bound $CO_2$ in the body fluids is about the same in cold and warm blooded animals, while the $O_2$ consumption per unit of time and body weight is much smaller in the former, and therefore metabolic disturbances producing an equal displacement of $CO_2$ produce disproportionate changes in R.Q. In any case we feel that the usual significance cannot be attached to the R.Q. in cold blooded animals unless the gaseous exchange has been determined continuously over a period of days or weeks. Where we consider the data technically correct and significant, we have included the R.Q. in the protocols.

The results obtained with Fish 41 indicate that as fasting proceeds and the metabolic energy comes to be furnished entirely by protein, the R.Q. approximates 0.840. (This is confirmed by a long series of postestivation data on Fish 12, not included in this report.) Hence the R.Q. of 0.801 obtained on Fish 217 at 20° indicates that fat is furnishing nearly all the energy not being supplied by protein. At the higher temperature the R.Q. is unquestionably aberrant owing to the displacement of $CO_2$ from the body fluids.

We had planned to make observations on the basal excretion of $SO_4$, $PO_4$, K, and creatine on normal fish which had never been in estivation, but the unexpected loss of all our active fish (which were stored at the New York Aquarium) prevented us from carrying out this program. We have, however, made a detailed study of one fish (No. 12) which had been fasted from 15 to 18 months, and which had been in estivation about 12 months. This fish was removed from estivation and returned to water on October 12, 1929. The accumulated products of metabolism were largely excreted in 13 days. For the next 4 months this fish was kept under observation, the excretion of various metabolites being carefully followed. At the end of this time it was apparently in a steady state and was excreting day by day such amounts of urea, $NH_3$,

$SO_4$, $PO_4$, creatine, and K as were concomitantly metabolized. At this time, because of the long period of fasting and the depletion of fat, 100 per cent of the energy of metabolism was being furnished by the combustion of protein. Over a period of 9 days at 20°, for each gm. of N excreted as urea and ammonia there were excreted simultaneously 2.84 mM of $SO_4$, 2.26 mM of Cl, 3.84 mM of $PO_4$, 5.05 mM of K, and 33.6 mg. of total creatinine N. These ratios may be somewhat aberrant because of the relatively emaciated condition of this specimen, but they will serve temporarily as a basis for comparison of the metabolism of these substances in the estivating fish.

### *Metabolism of Estivating Fish.*

We wished particularly to determine the metabolic rate of estivating fish under conditions as nearly natural as possible. We found that this could not be done with the fish encased in mud, so we removed them with as little stimulation as possible and placed them on cotton in the metabolism chamber. The course of metabolism during estivation and after estivation is terminated is illustrated by the data on Fish 12, 13, and 21 given in Protocols 1 to 3.

We find that, typically, the $O_2$ consumption is somewhat low on the 1st day after removal from mud, and that it rises on the 2nd or 3rd day to a level that is subsequently well maintained. At first sight it would appear that the metabolism might be increased by the stimulation of removing the fish from the mud. We do not believe this is the case, but think that the true estivating metabolism is represented by the level which is reached after the 1st day. If the metabolism were increased by the act of removing the fish from mud, one would expect the $O_2$ consumption to be high on the 1st day and to fall subsequently to a steady level. We have never observed a decrease in metabolism after the initial increase although some fish have been followed for 15 to 21 days. It is difficult to see why stimulation should lead to both a delayed and an essentially permanent increase. Moreover, we have not obtained a significant increase in $O_2$ consumption in estivating fish after handling or after such mild sensory stimulation as might be involved during removal from the mud.

Instead we believe that the low $O_2$ consumption observed on the 1st day is related to the extensive blowing off of $CO_2$ which occurs at this time. A characteristic feature of the postestivation metabolism is the high R.Q. of the first few days. This is obviously referable to an excessive $CO_2$ elimination following removal from the poorly ventilated burrow. We have some evidence that, within certain limits, the $O_2$ consumption is dependent on the partial pressure of this gas in the respired air. We believe the loss of $CO_2$ occurring on the 1st day causes apnea and anoxemia and that the latter is primarily responsible for the decreased $O_2$ consumption of the 1st day or so. With this interpretation, we take the ultimate steady level as indicating the $O_2$ consumption during estivation.

A summary of important data on estivating fish is given in Table V which is discussed later in this paper. The typical level of $O_2$ consumption in fish that have been estivating from 15 to 18 months is about 8.0 cc. per kilo per hour. This is in marked contrast to 20 cc. per kilo per hour found for the active Fish 217 and 41.

One of the first questions evoked by these data is, to what factors is this reduction of metabolism attributable? In considering possible causes, several factors suggest themselves, such as the absence of muscular activity, reduced circulatory and respiratory work, progressive emaciation, accumulation of metabolites, etc. We have found it possible to evaluate some of these by various observations and experiments on different fishes, and since the results are important for the interpretation of the metabolic picture as a whole, these observations have been brought together in the following paragraphs.

The $O_2$ consumption of excised turtle heart muscle was found by Vernon (1910) to be 0.00014 cc. per gm. per beat. Snyder's (1918) observation on the heat production of turtle heart muscle indicates an $O_2$ consumpton of about 0.0002 cc. per gm. per beat. Starling and Evans (1914) found in the dog heart-lung preparation 0.00033 cc. per gm. per beat and Rhode's (1912) data for the cat yield about 0.00025 cc. per gm. per beat. We observed the heart rate carefully in Fish 21 by noting the displacement of the center of gravity of the estivating fish while it was floating in water. Until the fish finally sank in the water the rate remained constant at three times per minute. The heart in *Protopterus* weighs less than 1 gm. per kilo of fish. Therefore, the heart of a 1 kilo fish would use on the basis of the above figures no more than 0.0002 × 180 × 1.0 or 0.036 cc. of $O_2$ per hour.

Since respiration occurs only once or twice an hour, while the heart beats at the rate of 180 times per hour, it is not to be expected that respiration would use more $O_2$ than the heart. But to check up on this point experimentally, we have noted the change in $O_2$ consumption associated with the marked increase in respiration caused by $CO_2$. These experiments were performed with Fish 21 while in the estivating state.

After a control or normal period of 6 days, during which the average $O_2$ consumption was 6.16 cc. per kilo per hour, the alkali was omitted from the metabolism chamber and the latter was filled with air enriched in $CO_2$. During the control period the respiration of the fish had been counted by noting the oscillation of a drop of toluene in a horizontal tube connected with the metabolism chamber. The fish was breathing at intervals longer than 30 minutes, and each respiration consisted of a single inhalation. After 7 days in the $CO_2$-rich atmosphere the respiration had increased to more than six times an hour, and each respiration was of a multiple type consisting of several inhalations varying from five to twelve in number. Even if it is admitted that each of these multiple inhalations does not involve as much muscular work as a single normal respiration, it is still permissible to suppose that the total respiratory work in the $CO_2$-rich atmosphere was at least 10 times as great, and probably more, than in the previous $CO_2$-free period. Under these conditions of hyperpnea the $O_2$ consumption increased from 6.16 to 7.56 cc. per kilo per hour with the value 7.26 cc. per kilo per hour on the last day when the respiratory effort was greatest. Therefore, a difference of 1.10 cc. per kilo per hour represents the maximum oxygen which could be assigned to the respiratory efforts when increased at least 10-fold, and it is doubtful whether all of this increase is necessarily attributable to this cause. By using this figure as the maximum value, the normal respiratory work during estivation would consume no more than one-tenth as much, or 0.11 cc. per kilo of fish per hour.

The total $O_2$ referable to circulation and respiration would then not exceed 0.036 + 0.11 cc., or 0.14 cc. per kilo of fish per hour.

Since the estivating fish is perfectly quiet and probably entirely devoid of muscular tone, the $O_2$ not referable to circulation and respiration must go to what we may call non-mechanical processes. If 8 cc. per kilo per hour are taken as the typical $O_2$ consumption of estivating fish, it is clear that about 7.9 cc., or 98 per cent, are referable to such non-mechanical metabolism.

When the estivating fish is returned to water it awakens and is forced to swim to the surface in order to breathe. There is concomitantly a slight increase in $O_2$ consumption which is presumably largely attributable to this renewed muscular activity. The

*Protocol 1, Fish 12.*

The fish was fasted from July 15, 1928. Placed in mud September 28, 1928; weight 248.15 gm. Removed from mud, October 24, 1929. Estimated dry estivation 358 days; weight 207.0 gm. The fish was dormant but showed frequent respiration. It was placed in the metabolism chamber with NaOH solution. The temperature was 19–21°.

| Date. | $O_2$ | R.Q | Mg. per kg. per day. | | | | mM per kg. per day × $10^3$. | | | Respirations per hr. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total N. | Urea + $NH_3$-N. | $NH_3$-N. | Total creatine N. | $SO_4$ | $PO_4$ | K | |
| *1929 Oct.* | *cc. per kg. per hr.* | | | | | | | | | |
| 25 | 4.0 | 2.38 | | | | | | | | |
| 26 | 5.5 | 0.96 | | | | | | | | |
| 27 | 6.6 | 0.70 | | | | | | | | |
| 28 | 4.1 | 0.81 | | | | | | | | |
| 29 | 6.8 | 0.75 | | | | | | | | |
| 30 | 6.5 | 0.67 | | | | | | | | <2 |
| 31 | 7.6 | 0.56 | | | | | | | | <2 |
| *Nov.* | | | | | | | | | | |
| 1 | 6.2 | 0.61 | Alkali omitted and 1% $CO_2$ put in chamber. | | | | | | | <2 |
| 2 | 9.2 | 0.32 | " " " 2% " " " " | | | | | | | <4 |
| 3 | 6.8 | 0.57 | " " " 2% " " " " | | | | | | | <4 |
| 4 | 7.4 | 0.33 | " " " 3% " " " " | | | | | | | >4 |
| 5 | 6.4 | 0.66 | " " " 4% " " " " | | | | | | | >4 |
| 6 | 7.3 | 0.34 | " " " 4.6% " " " " | | | | | | | >6 |
| 7 | 8.3 | 0.83 | $CO_2$ omitted and alkali returned to " | | | | | | | 1 |
| 8 | 5.2 | 0.96 | | | | | | | | |
| 9 | 5.7 | 0.84 | | | | | | | | |
| 11 | 5.6 | 0.78 | | | | | | | | |
| 12 | 6.6 | 0.78 | | | | | | | | |

*Protocol 1—Concluded.*

Fish placed in 1000 cc. of water for 1 hour to wash, then transferred to 500 cc. of water in metabolism chamber; weight 204 gm.

| Date. | $O_2$ | R.Q. | Mg. per kg. per day. | | | | mM per kg. per day × $10^3$. | | | Respirations per hr. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total N | Urea + $NH_3$-N | $NH_3$-N. | Total creatine N. | $SO_4$ | $PO_4$ | K | |
| *1929* | *cc. per kg per hr.* | | | | | | | | | |
| Wash solution.. | | | 328 | 296 | 37.0 | 0.74 | 4450 | 18.1 | 26 | |
| *Nov.* | | | | | | | | | | |
| 13 | 10.7 | 0.98 | 2040 | 1970 | 7.9 | 0.49 | 662 | 17.1 | 1330 | |
| 14 | 11.2 | 0.70 | 1670 | 1650 | 9.6 | 0.50 | 1150 | 2.5 | 1122 | |
| 15 | 11.7 | 0.71 | 870 | 845 | 19.7 | 0.73 | 1051 | 25.5 | 795 | |
| 16 | 12.2 | 0.87 | 321 | 296 | 17.6 | 0.74 | 980 | 46.5 | 191 | |
| 17 | 13.5 | 0.81 | 164 | 133 | 18.0 | 0.74 | 613 | 66.0 | 216 | |
| 18 | 9.3 | 0.87 | 72.0 | 49.3 | 12.3 | 0.74 | 416 | 74.0 | 79 | |
| 19 | 10.2 | 0.89 | 67.7 | 41.4 | 11.5 | 0.74 | 294 | 85.0 | 93 | |
| 20 | 11.3 | 0.83 | 62.0 | 47.5 | 14.9 | 1.22 | 294 | 117 | 39.2 | |
| 21 | 11.3 | 0.84 | 55.5 | 39.0 | 18.5 | 0.98 | 245 | 173 | 88.2 | |
| 22 | 11.7 | 0.83 | 45.3 | 29.0 | 16.8 | 0.98 | 157 | 204 | 215.8 | |
| 23 | 10.1 | 0.87 | 32.7 | 23.2 | 14.8 | 0.98 | 98.0 | 232 | 328.0 | |
| 24 | 8.9 | 0.92 | 36.9 | 21.2 | 14.5 | 0.97 | 88.3 | 255 | 117.8 | |
| 25 | 8.0 | 0.92 | 44.6 | 20.8 | 14.3 | 0.74 | 63.6 | 222 | 166.6 | |
| Average. | 10.8 | 0.85 | | | | | | | | |
| Total. | | | 5814 | 5461 | 227.4 | 11.30 | 10,560 | 1537 | 4807 | |

postestivation metabolism of Fish 21, on which the above $CO_2$ experiments were performed, was not measured, but data are available on three other fish. In the case of Fish 12 the $O_2$ consumption rose from the estivating level of 6.5 cc. per kilo per hour to 9.6 cc. after the fish was returned to water. In the case of Fish 13, the $O_2$ consumption increased from 12.3 cc. during estivation to an average of 14.9 after recovery. In Fish 17 (the data on which we are not reporting in full) these figures were 19.6 and 22.4 cc., respectively. Since the metabolism of these fish in the postestivation period includes the muscular activity associated with body movements, increased respiration, circulation, muscular tone, etc., which characterized the normal fish (Nos. 217 and 41) when kept under the same conditions, this increase may be taken as the $O_2$ normally used by these mechanical processes. This increase in Fish 12, 13, and 17 averages 2.8 cc. of $O_2$ per kilo per hour.

We are led to conclude, then, that out of the 20 cc. of $O_2$ per kilo per hour used by the active fish, no more than 3.0 cc. are referable to muscular activity and tone and to other increased physiological processes related to the active condition. Therefore the remaining 17 cc. must go to the non-mechanical or maintenance processes. Since the estivating $O_2$ may fall as low as 5 cc. per kilo per hour, it follows that a reduction of 12 cc., or 70 per cent, may occur in this non-mechanical metabolism.

*Protocol 2, Fish 13.*

The fish was fasted from July 15, 1928. Placed in mud September 28, 1928; weight 228.05 gm. Removed from mud November 7, 1929. Estimated dry estivation 390 days; weight 173.3 gm.

| Date. | $O_2$ | R Q |
|---|---|---|
| *1929* | *cc. per kg per hr.* | |
| Temperature 19–20°. | | |
| Nov. 8 | 7.9 | (1.40) |
| " 9 | 6.3 | (1.01) |
| " 10 | 7.2 | 0.77 |
| " 11 | 7.2 | 0.77 |
| " 12 | 8.4 | 0.71 |
| " 13 | 7.3 | 0.85 |
| " 14 | 8.7 | 0.84 |
| " 15 | 8.7 | 0.83 |
| " 16 | 8.8 | 0.73 |
| Average | 7.8 | 0.79 |
| Temperature 30°. | | |
| Nov. 17 | 27.2 | 0.73 |
| " 18 | 25.5 | 0.91 |
| " 19 | 29.1 | 0.85 |
| " 20 | 25.4 | 0.89 |
| " 21 | 21.5 | 0.95 |
| " 22 | 21.2 | 0.87 |
| " 23 | 20.8 | 0.91 |
| " 24 | 20.8 | 0.99 |
| " 25 | 19.2 | 1.09 |
| Average | 23.4 | 0.91 |

| Date. | $O_2$ | R Q. |
|---|---|---|
| *1929* | *cc. per kg. per hr.* | |
| Temperature 18–20°. | | |
| Nov. 26 | 5.5 | 1.22 |
| " 27 | 7.6 | 0.82 |
| " 28 | 8.5 | 0.88 |
| " 29 | 8.5 | 0.88 |
| " 30 | 8.4 | 1.07 |
| Average Dec. 1–7 | 11.3 | 0.97 |
| 0.01 mg. adrenalin intramuscularly. | | |
| Dec. 8 | 9.5 | 0.97 |
| " 9 | 10.2 | 0.88 |
| " 10 | 8.4 | 1.16 |
| 0.01 mg. adrenalin intramuscularly. | | |
| Dec. 11 | 10.4 | 0.93 |
| " 12 | 10.9 | 0.88 |
| 0.02 mg. adrenalin intramuscularly. | | |
| Dec. 13 | 10.4 | 0.89 |
| " 14 | 12.3 | 0.82 |

*Protocol 2—Concluded.*

December 15, accident in water bath broke metabolism chamber and let water in on fish, necessitating termination of estivation. No wash solution. Total duration of estivation, 410 days; weight 168 gm. Fish placed in 500 cc. of water in metabolism chamber. The temperature was 20°.

| Date. | $O_2$ | R.Q. | Mg. per kg. per day. | | | | mM per kg. per day. | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total N. | Urea + $NH_3$-N. | $NH_3$-N. | Total creatine N. | $SO_4$ | $PO_4$ | Cl | K |
| *1929* | *cc. per kg. per hr.* | | | | | | | | | |
| Dec. 16 .... | | | 480 | 412 | 19 | 0.72 | 685 | 27 | 1730 | 910 |
| " 17 .... | 11.3 | 0.89 | 1005 | 897 | 24 | 1.09 | 1250 | 48 | 3110 | 535 |
| " 18 ... | 14.8 | 0.92 | 1080 | 1023 | 47 | 1.37 | 1053 | 83 | 2050 | 478 |
| " 19 .... | 14.3 | 0.97 | 885 | 848 | 53 | 1.48 | 849 | 109 | 1590 | 242 |
| " 20 . | 15.1 | 0.88 | 815 | 743 | 50 | 1.18 | 612 | 148 | 810 | 196 |
| " 21..... | 15.5 | 0.85 | 582 | 545 | 29 | 0.89 | 398 | 164 | 350 | 37 |
| " 22 ... | 15.3 | 0.85 | 416 | 366 | 33 | 0.72 | 312 | 222 | 0 | 3 |
| " 23 .... | 14.3 | 0.85 | 346 | 302 | 32 | 0.69 | 265 | 254 | 0 | 15 |
| " 24 ... | 13.0 | 0.92 | 275 | 240 | 32 | 0.74 | 196 | 300 | 0 | 33 |
| " 25..... | | | 147 | 141 | 13 | 1.08 | 185 | 300 | 0 | 22 |
| " 26 .... | 13.3 | 0.87 | 128 | 116 | 17 | 1.15 | 208 | 415 | 0 | 1 |
| Average.. | 14.1 | 0.89 | | | | | | | | |
| Total..... | | | 6159 | 5633 | 349 | 11.11 | 6013 | 2070 | 9640 | 2472 |

Because of infection, the fish was retired to $KMnO_4$ bath; died December 28. No visible fat in tail muscles or viscera.

When we inquire as to the cause of this reduced metabolism, we note first that apart from the 3 cc. increase attributable to resumed muscular activity, the metabolism remains at this low level after all accumulated metabolites have been excreted from the body. The estivating metabolism is, moreover, apparently independent of the quantity of these metabolites in the body; thus, Fish 7 contained 2.55 per cent urea and used 15 cc. of $O_2$ per kilo per hour, which is almost as much as the normal fish, while Fish 15, with only 1.60 per cent urea, used only 5 cc. of $O_2$ per kilo per hour (*cf.* Table V). These two lines of evidence make it improbable that the reduced metabolism is due to any inhibitory influence of the accumulated urea or other metabolites. The persistence of the low metabolic rate after estivation is terminated likewise rules out any inhibitory influence of a nervous origin, for the fish is at this time in a state of normal reactivity.

The evidence points, instead, to the emaciated condition of the *animal as the primary cause of lowered metabolism.* Though there is no correlation among our data between the extent of tissue destruction (as indicated by the accumulated urea) and metabolic rate, still this fact cannot be held to disprove this interpretation. It may be that all nitrogenous tissue is not equipotential in contributing to metabolic activity. There may be, moreover, con-

*Protocol 3, Fish 21.*

The fish was fasted from July 15, 1928. Placed in mud September 28, 1928; weight 181.7 gm. Removed from mud November 25, 1929. Estimated dry estivation 390 days; weight 133.4 gm. The temperature was 20°.

| Date. | $O_2$ | R Q. | $O_2$ in chamber. |
|---|---|---|---|
| *1929* | *cc per kg per hr* | | *per cent* |
| Nov. 26 | 5.1 | 3.63 | 21.0 |
| " 27 | 7.5 | 1.28 | 21.0 |
| " 28 | 8.5 | 0.94 | 21.0 |
| " 29 | 8.5 | 0.94 | 21.0 |
| " 30 | 7.4 | 1.00 | 21.0 |
| Dec. 1 | 8.8 | 0.93 | 21.0 |
| " 2 | 8.8 | 0.93 | 21.0 |
| " 3 | 7.8 | 1.13 | 21.0 |
| " 4 | 8.4 | 0.97 | 21.0 |

Chamber filled at beginning with nitrogen-air mixture, with $O_2$ as shown.

| Date. | $O_2$ | R Q. | $O_2$ in chamber. |
|---|---|---|---|
| Dec. 5 | 12.2 | 0.73 | 11.4 |
| " 6 | 12.0 | 0.77 | 10.7 |
| " 7 | 11.5 | 0.85 | 11.4 |
| " 8 | 9.6 | 0.91 | 11.4 |
| " 9 | 9.0 | 0.91 | 11.3 |
| " 10 | 8.1 | 1.05 | 11.5 |

Chamber refilled with air.

| Date. | $O_2$ | R Q. | $O_2$ in chamber. |
|---|---|---|---|
| Dec. 11 | 10.7 | 0.75 | 21.0 |
| " 12 | 7.2 | 1.03 | |
| " 13 | 9.8 | 0.78 | |

| Date. | $O_2$ | R Q. |
|---|---|---|
| *1929* | *cc. per kg per hr.* | |
| Average Dec. 13–23 | 8.7 | 0.85 |

December 23, 0.5 mg. of crystalline thyroxine (Squibb) was injected intramuscularly; weight 125.9 gm.

| Date. | $O_2$ | R Q. |
|---|---|---|
| Dec. 24 | 8.3 | 0.97 |
| " 25 | 8.1 | 0.97 |
| " 26 | 10.7 | 0.77 |
| " 27 | 11.4 | 0.84 |
| " 28 | 14.4 | 0.78 |
| " 29 | 14.3 | 0.88 |
| " 30 | 13.0 | 0.91 |
| " 31 | 14.9 | 0.87 |
| *1930* | | |
| Jan. 1 | 13.5 | 1.03 |
| " 2 | 13.5 | 1.03 |
| " 3 | 17.0 | 0.85 |
| " 4 | 16.5 | 0.89 |
| " 5 | 16.9 | 0.63 |
| " 6 | 16.9 | 0.63 |

*Protocol 3—Concluded.*

Fish infected. Awoke and broke out of cocoon; weight 121.2 gm. Removed, washed 30 min., and put in 500 cc. of water in metabolism chamber at 20°.

| Date. | Weight | Mg. per kg. per day. | | | | mM per kg. per day $\times 10^3$. | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Total N. | Urea + $NH_3$-N. | $NH_3$-N. | Total creatine N. | $SO_4$ | $PO_4$ | Cl | K |
| *1930* | *gm.* | | | | | | | | |
| Wash solution with cotton on which fish had been kept.............. | | 750 | 726 | 32.2 | 1.24 | 3785 | 206 | 1650 | 923 |
| Jan. 7...... | 133 2 | 5500 | 5100 | 33.0 | 1.12 | 446 | 49 | 1280 | 48 |
| " 8 . ... | 145 6 | 1230 | 1196 | 48 5 | 2 80 | 1360 | 114 | 2230 | 158 |
| " 9 .... | 153 5 | 412 | 354 | 41.2 | 7.50 | 2000 | 154 | 1570 | 49 |
| " 10 ... | 153 5 | 269 | 229 | 43.4 | 9 90 | 2048 | 132 | 1152 | 92 |
| " 11...... | 149 7 | 160 | 129 | 33 0 | 9.90 | 1403 | 229 | 1402 | 49 |
| " 12 ..... | 146 3 | 135 | 81 | 34.4 | 5 48 | 1040 | 393 | 1302 | None. |
| " 13...... | 144.0 | 87 | 35 | 23.5 | 4.12 | 617 | 330 | 1480 | 73 |
| " 14 ..... | 148 4 | 82 | 52 | 23 5 | 4.12 | 617 | 338 | 3300 | 520 |
| " 15. .... | 152 0 | 110 | 43 | 26 6 | 4.00 | 400 | 250 | 800 | 100 |
| Total.. ... .... | | 8735 | 7945 | 339.3 | 50.2 | 13,716 | 2195 | 16,166 | 2012 |

Because of the severity of the infection the fish was sacrificed. No visible fat was present.

tributory factors, such as endocrine secretion, which are profoundly modified as emaciation develops, and these may be affected to different degrees in different individuals.

It is convenient to note here that the degradation of protein N to urea (see below) indicates that protein continues to furnish a large fraction of the metabolic energy in the estivating animal. As the fat is used up, this fraction must rise to 100 per cent, as we have observed in two fish (Nos. 41 and 12). It does not appear that the protein fraction is ever much less than 50 per cent even in fish fasted but a short time. In relation to the gross economy of the animal this high protein combustion greatly reduces the number of calories which can be obtained from each gm. of body weight lost during fasting. Thus where 50 per cent of the metabolic energy is derived from protein, as in the case of *Protopterus* and possibly cold blooded animals in general, each gm. of weight lost supplies only about 1.6 calories as compared with the theoretical 9.3 calories that might be obtained from 1 gm. of fat. In man and other mammals protein furnishes only 15 per cent of the energy, hence each gm. of weight lost supplies about 4.0 calories. Thus mammals are more efficient candidates for fasting than *Protopterus* by a ratio of 2.5:1. If we suppose that the maximal duration of a fasting period were limited to the loss of an equal fraction of the body weight, mammals would be good for 2½ times as many calorie hours as the lung-fish. It seems possible that this difference in the relative amounts of fat and protein burned during fasting depends upon differences in capacity to store, transport, and utilize fat, and that in general a greater utilization of fat may be one of the evolutionary concomitants of homeothermy.

In any case, it is clear that *Protopterus* meets the emergency of indefinite fasting principally by reducing the truly basal, or non-mechanical, metabolism to progressively lower levels as time goes on. The fish listed in Table V have burned in 380 days an average of 0.884 gm. of N out of an initial 4.4 gm. (182.3 gm. × 2.4 per cent) of protein N, or 20 per cent. Because of the lowered metabolic rate in the emaciated animal, we may suppose that another and equivalent quantity of protein N would serve for at least twice as long, so that a conservative estimate would put the period of possible fasting at 3 to 4 years, and if the metabolic rate continues to decrease, as it may, this estimate would have to be put at a still longer and more indefinite time.

### *Effect of Temperature, Thyroxine, Etc., on Estivating Fish.*

We wished to observe the effects of an increase in temperature on the metabolism of estivating fish for two reasons; first, to determine whether temperature would have its customary effect of increasing the metabolism, and second, in the event that an increase occurred, to observe whether the state of sleep would be modified by it.

Fish 13 was accordingly transferred to 30° temperature after a preliminary 9 day control period at 20°. The $O_2$ consumption rose immediately from 7.82 to 27.2 cc. per kilo per hour. This increase was relatively greater than was observed under the same conditions in the active fish (No. 217, 20 cc. at 20° to 36 to 41 cc. at 30°). There can be no doubt that in estivating fish the metabolism shows the usual increase under the influence of a higher temperature, and falls again to its typical, low value when the temperature is again reduced. This fact supports the view that the lowered metabolism of estivation is not due to active inhibition.

It was particularly noted that at the higher temperature, when the metabolic rate was 3 to 4 times that prevailing during estivation, the animal remained perfectly quiescent. There were no spontaneous movements and no apparent increased reactivity. This fish had always been in a state of deepest inhibition, and continued so throughout the 9 days during which it was kept at 30° and for 12 days thereafter. The metabolic rate of this fish at 30° was greater by 300 to 400 per cent than the active level of Fish 217. It is to be concluded, therefore, that the state of sleep which obtains during estivation is not due to the lowered metabolic rate.

Temperature as a cause of sleep is clearly ruled out for the reason that Fish 13 remained asleep at 30°, while all the fish entered into estivation at the temperature (20°) at which they normally live and remain active. As will be seen later, when returned to water the animal is awakened in a few hours, apparently by developing asphyxia, and soon becomes as active as a normal fish. Since the accumulated products of metabolism require several days for their excretion, during which time the fish is fully active, the state of sleep cannot be attributed to the presence of these substances in the body. The estivating fish were kept under observation in the saturated atmosphere of the metabolism chamber for as long as 6 weeks without showing any signs of awakening ascribable to this circumstance, and data to be presented at another time show that there is little, if any, desiccation of the tissues during estivation. Therefore we conclude that dehydration plays no part in the induction of the inhibited state.

With these factors ruled out we are reduced to the assumption that the state of sleep is induced by the continued immobilization of the animal, supplemented, perhaps, by sensory stimulation issuing from contact with the rigid walls of the mud nest and by the withdrawal of free water. We have kept active fish for 6 days in moist aquaria without water with no apparent inhibition, so the withdrawal of water appears to be only slowly effective, if this factor plays any part at all in this phenomenon.

To return to the question of the control of the metabolic rate, there is an obvious possibility that endocrines are normally operating to maintain the non-mechanical metabolism and that during estivation their influence is removed or reduced in intensity. This supposition led us to observe the effects of thyroxine on estivating fish.

The effects of thyroxine were observed in Fish 21. This fish has been followed in estivation for 19 days, during part of which time it was kept in an atmosphere of reduced $O_2$ concentration. This fact did not affect the metabolic rate and there is no reason to believe that it altered the subsequent results. After several days of fairly constant metabolism, 0.5 mg. of crystalline thyroxine, kindly supplied us by E. R. Squibb and Sons, was administered in solution by intramuscular injection. Prior to the injection the $O_2$ consumption had been about 8 cc. per kilo per hour. No effect was noted during the first 2 days after the injection. On the 3rd day the $O_2$ consumption began to increase, and it continued in an upward direction for 9 days, reaching a maximum of 17.0 cc. per kilo per hour, or over twice the previous level. For 7 days the $O_2$ consumption was at or above the level of 14.0 cc. per kilo per hour. The quantity of thyroxine administered is admittedly large, amounting to 27.6 mg. per kilo, but this substance appears to be less effective in cold blooded animals than in man. That it had a positive effect in increasing metabolism is beyond question, and it is worth while noting that the slow and prolonged action characteristic of its effects in man are in evidence. It is also noteworthy that this animal showed no tendency to waken under the influence of the hormone. Ultimately infection developed and spontaneous movements appeared which made it advisable to terminate estivation. But for 8 days, during which time the $O_2$ consumption was 75 per cent above the estivating level, the fish was perfectly quiescent. This fact confirms our previous conclusion that increased metabolism does not waken the animal, and in addition, permits us to conclude that thyroxine does not have a specific action in this direction.

Adrenalin was administered by intramuscular injection to Fish 13 without effect, though this negative result is not conclusive since the dosage was relatively small.

In considering the circumstances under which the fish is naturally wakened, it is to be noted that merely placing the fish in water is without effect. The animal floats until such time as the air is expelled from the lungs, after which it sinks quietly to the bottom. The fish is unable, of course, to obtain air under water and, if the cocoon is removed from the head, the mouth may be observed to open and close at increasingly frequent intervals as respiratory distress becomes augmented. Ultimately, muscular movements of increasing force appear until at last the fish breaks free and struggles to the surface. From this evidence it would appear that the development of asphyxia was the factor that wakened the animal. It was observed in experiments with Fish 12 that $CO_2$ in concentrations of 3 to 4 per cent has no effect upon the fish except to increase respiration. Fish 21 was kept for 5 days in $N_2$-air mixtures having initial $O_2$ concentrations ranging from 10.66 to 11.47 per cent. Apart from a slight, incidental increase in $O_2$ consumption during the first 3 days, the lowered $O_2$ concentration was without effect. It is entirely possible, however, that a greater degree of asphyxia develops after immersion in water than would be represented by 11.0 per cent of $O_2$ or 4.0 per cent of $CO_2$ applied separately.

### *Excretion of Accumulated Metabolites.*

It has been stated above that all the evidence points to the complete cessation of urine excretion during estivation. This conclusion is amply confirmed by the analyses of the estivating fish, and by the relatively enormous excretion of urea after the fish is returned to water.

The excretion of various metabolites accumulated during estivation has been carefully followed in several fish. Detailed data are given in Protocols 1, 2, and 3 for Fish 12, 13, and 21 respectively.

Fish 12 and 21 were removed from mud with the cocoon nearly intact, and when estivation was terminated they were washed in a suitable volume of water for 1 to 3 hours to remove any urea, etc., which might be in the cocoon or on the skin. In these and several other instances the mud opposite the cloaca was analyzed and compared with mud from the top of the jar. In no case could significant quantities of urea or of other nitrogenous substances be recovered from this mud. Small quantities of urea and other metabolites can be recovered from the water in which the fish is

TABLE III.

*Composition of Muscle Tissue of Protopterus.*

| | | Fish 33, control. | Fish 34, control | Fish 5, estivating | Fish 15, estivating. | Fish 8, estivating | Fish 13, recovered. | Fish 21, recovered. |
|---|---|---|---|---|---|---|---|---|
| $H_2O$, | *per cent* | 81.8 | 82.0 | 78.5 | 81.4 | 78.5 | 87.0 | 88.2 |
| Total N | " " | 2.62 | 2.79 | 3.62 | 3.23 | | 1.90 | 1.85 |
| Protein N | " " | 2.35 | 2.46 | 1.99 | 1.89 | | 1.65 | 1.63 |
| Non-protein N | " " | 0.271 | 0.333 | 1.630 | 1.375 | 0.543 | 0.250 | 0.216 |
| Urea + $NH_3$-N | " " | 0.013 | 0.015 | 1.120 | 0.871 | 0.340 | 0.040 | 0.082 |
| $NH_3$-N | " " | 0.004 | 0.007 | 0.006 | 0.006 | 0.005 | | |
| Total creatine N | " " | 0.149 | 0.147 | 0.183 | 0.171 | 0.147 | 0.137 | 0.124 |
| Uric acid N | " " | 0.0001 | 0.0001 | | 0.0001 | 0.0001 | | |
| Glycogen | " " | | | | | 0.170 | | |
| $PO_4$, | *mM per kilo* | 48.0 | 41.6 | 53.8 | 51.6 | 98.0 | 48.1 | 45.0 |
| $SO_4$ | " " " | Trace. | 0.9 | | | | | |
| K | " " " | 62.2 | 58.8 | 51.8 | 69.7 | 69.5 | 65.4 | 58.9 |
| Corrected to normal water content. | | | | | | | | |
| Total N, | *per cent* | 2.62 | 2.79 | 3.43 | 3.30 | | 2.66 | 2.87 |
| Corrected protein N | " " | 2.35 | 2.46 | 1.89 | 1.93 | | 2.32 | 2.52 |
| " total creatine N | " " | 0.149 | 0.147 | 0.173 | 0.175 | 0.128 | 0.192 | 0.194 |

washed. These represent traces accumulated on the skin and cotton owing to resumption of kidney excretion in the moist chamber, and in part, traces excreted while the fish is in the wash solution. But the amounts of urea, etc., which can be recovered from the cocoon and from the outside of the fish are quite small in comparison with the quantities that are excreted during the first 24 hours in water.

With the resumption of aquatic life the urea is excreted in spectacular quantities. That this urea is present in the estivating fish prior to its reversion to aquatic life is shown by the analyses of the muscles given in Table III; the urea content of the muscles may increase well over 100 times during estivation. Moreover, the failure of the $O_2$ consumption to show any considerable increase during the postestivation period shows that the urea which is excreted at this time is not to any significant degree formed concurrently by stimulated protein metabolism.

*Protopterus* does not have a urinary bladder, and we have never found significant quantities of fluid or of urea accumulated in the cloaca. In the one fish in which the analyses were made (Fish 8), urea was found in the same concentration in the bile and in the blood. There is no evidence that urea can be concentrated by fish in any excrement, so it would appear that most of this excreted urea is actually removed from the blood and tissues concurrently with its observed excretion. This conclusion is supported by the rate of its excretion. Typically (Fish 12 and 13, and Fish 29, 9, and 17 which are not reported in detail) the quantities excreted reach a maximum on the 2nd or 3rd day and decrease gradually through 8 to 12 days to the basal level. In one instance, however, (Fish 21) there was a relatively enormous and maximal excretion during the first 24 hours, amounting to 5066 mg. of urea N per kilo. If this quantity of urea were excreted unconcentrated in urine or some fluid it would have required 670 cc. of water per kilo of fish to carry it out of the body—an improbable volume. We presume that this urea is excreted largely by diffusion across the gills. If we consider all the recovered urea as initially dissolved in the body water, and if we take this to be 82 per cent of the body weight, we would have in Fish 21 a total of $\frac{7606}{0.82}$ or 928 mg. of urea N per cent. This fish was kept in 1000 cc. of water which at the end of the first 24 hours had a urea N concentration of 61.6 mg. per cent. Thus a terminal concentration gradient of about 15:1 existed between the body fluids and the external solution. Calculated in the same manner, this gradient in Fish 12 and 13, which were kept in 500 cc. of water, was 8 and 21, respectively. So even if considerable error is allowed for, there is no doubt that a sufficient concentration gradient exists between the blood and the external water in all our experiments to permit the excretion of the urea by diffusion.

The data given in Table II show that the fasted but active lungfish excretes from 30 to 70 per cent of its nitrogen as $NH_3$. In view of this fact it is important to note that nitrogen accumulates in the body during estivation entirely as urea, and that it is excreted as such after estivation is terminated. The ammonia excretion is never significantly increased, but begins at a level which is comparable with that of the normal fish (*i.e.*, 20 to 40 mg. per kilo per day). The fact that no $NH_3$ accumulates during estivation may be related to the peripheral formation of this substance, as in the mammals, or it may be related to the suspension of muscular activity or some other phase of internal metabolism.

The urea and $NH_3$-N make up most of the total N excreted after estivation. This sum constitutes in Fish 12, 94 per cent of the total N and 91 per cent in Fish 13 and 21. Unquestionably a considerable fraction of the residuum is mucin N in the slime, which is quite abundantly excreted at this time. Slime excretion was minimal in Fish 12, possibly because this fish remained entirely free of infection, and therefore we believe that the higher figure obtained in this case is significant. It would appear, then, that during estivation at least 94 per cent (if not all) of the protein N is degraded either to urea or to an ammonia precursor.

Coincident with the excretion of urea there occurs a rapid excretion of $SO_4$. After passing through an early maximum the daily excretion falls rapidly toward the basal level. The accumulation of $SO_4$ during estivation is to be expected from the oxidation of protein sulfur. The rapid excretion of this substance, which we believe to be excreted entirely by the kidneys in fish (Smith, 1930,*a*), shows that renal activity is rapidly resumed.

The $SO_4$ excretion was essentially at the basal level within 10 days in Fish 12, 13, and 21, from which fact we reason that the kidneys should be capable of excreting any other accumulated metabolic products within this period of time. In view of this fact, it is particularly significant that there is no increased excretion of creatine and $PO_4$ after the termination of estivation; their excretion begins with very small amounts and rises slowly over a period of days to the basal level. The fact that urea and $SO_4$ are massively excreted after estivation, while creatine and $PO_4$ are not excreted at this time in even normal amounts, suggests that there is an important difference in the metabolism of these two groups of substances.

In order to evaluate these results more accurately, we made a careful series of observations on the basal excretion of N, $SO_4$, etc., in Fish 12, as described above. By using the basal ratios obtained in this fish we have calculated from the recovered N in Fish 12, 13, and 21 how much $SO_4$, etc., should be excreted had these substances accumulated in the body during estivation in amounts proportional to the nitrogen. These calculations are given in Table IV in comparison with the actual quantities of each metabolite recovered.

TABLE IV.

*Comparison of Metabolites Recovered and Expected from Estivating Fish.*

| | Fish 12. | | Fish 13. | | Fish 21. | | Average per cent recovered. |
|---|---|---|---|---|---|---|---|
| | Recovered. | Expected. | Recovered. | Expected. | Recovered | Expected. | |
| Urea + $NH_3$-N, *mg* | 5461 | | 5633 | | 7945 | | |
| $SO_4$, *mM* | 10.6 | 15.5 | 6.0 | 16.0 | 13.7 | 22.6 | 55.5 |
| Cl " | | | 9.6 | 12.7 | 16.1 | 12.8 | 100.0 |
| Total creatine N, *mg* | 11.3 | 184.0 | 11.1 | 189.0 | 50.2 | 267.0 | 10.3 |
| $PO_4$, *mM* | 1.5 | 21.0 | 2.1 | 21.6 | 2.2 | 30.5 | 8.0 |
| K " | 4.8 | 27.6 | 2.5 | 28.4 | 2.0 | 40.1 | 10.4 |

Reference to these data shows that about 50 per cent of the expected $SO_4$ is recovered simultaneously with the nitrogen. The lowest recovery is from Fish 13 from which the wash solution was lost; since a considerable amount of $SO_4$ was excreted by Fish 12 and 21 in the urine formed in the moist air of the metabolism chamber, we think the lower recovery in Fish 13 was attributable to this fact. There is every reason to believe that if $SO_4$ can be excreted by the kidney, creatine and $PO_4$ can also be excreted. Yet in these same periods (9 to 13 days) there were excreted by these fish only 10 per cent of the expected creatine, 8 per cent of the expected $PO_4$, and 10 per cent of the expected K. This small recovery, coupled with the ascendant character of the excretion curves, indicates that the metabolism of these substances is only gradually resumed after estivation is terminated.

The metabolism of creatine and creatinine has not been examined extensively in the lower animals. More creatine than creatinine is normally excreted by birds, turtles, and the python (Hunter, 1928). Dorner (1907), van der Heyde (1921), and Hsu (1925) were unable to find either substance in the urine of frogs, though this failure was possibly attributable to the very dilute nature of the urine examined. (As judged by the Jaffe reaction we have rarely failed to find either creatine, creatinine, or both in the urine of fasted and fed frogs in metabolic studies including a large number of observations (unpublished).) Creatine predominates over creatinine in the urine of fish (Denis, 1913–14; Marshall and Grafflin, 1928; Grollman, 1929; Smith, 1929; Marshall, 1930). It is noteworthy that Grollman isolated creatine from *Lophius* urine and showed that the Jaffe reaction gives results which are in good agreement with the specifically identified yield of creatine. We have found in the urine collected from freshly caught specimens of *Protopterus* traces of creatine but no creatinine (see Table I). The fact that ammonia and urea are excreted by the gills in fish leads to the creatine assuming a disproportionate fraction of the total urinary nitrogen (Smith, 1929), though when the extrarenally excreted nitrogen is added to the urinary constituents the total creatine approximates the usual proportion of the total nitrogen (*i.e.*, 2 to 6 per cent). From these facts it appears that creatine and creatinine N is largely excreted in the first form by fish, but apart from this fact there is no evidence that these substances have any other significance in these animals than in mammals.

The above evidence might be interpreted as showing either that creatine metabolism is suspended in the lung-fish during estivation, or that this substance is anabolized in a cyclical fashion as fast as it is catabolized. We see no way to decide between these alternative explanations, but it is significant that the estivating fish is completely immobilized in its mud nest; not only is muscular activity (except as related to respiration) impossible, but the conditions are such as to suggest that muscular tone is also in complete abeyance.

Some importance attaches to the fact that $PO_4$ behaves like creatine, in that there is no apparent accumulation of this substance during estivation, although large amounts are excreted by the active, fasted fish. Fiske and Subbarow (1929) have shown that there normally exists in the skeletal muscles of mammals a labile phosphocreatine compound which during muscular activity or after injury decomposes to yield creatine and inorganic phosphate. Our observations on the lung-fish lead us to suggest that the simultaneous abeyance of both creatine and phosphate catabolism during estivation issues from the natural chemical relationship between these substances in the muscle.

The excretion of K is much less in the postestivating fish than would be expected from the basal N:K ratio in active fish. Such K as is excreted, however, escapes from the body rapidly at first and in diminishing amounts thereafter. In this respect K differs from creatine and $PO_4$ since it appears to accumulate to a slight extent in a catabolized state during estivation. The amounts thus excreted are so small, however, that one is led to suspect that the metabolism of this substance in estivating fish is in part, at least, related to the metabolism of creatine and $PO_4$.

The above facts lead us to divide the metabolism of the lung-fish into two categories: one pertaining to the combustion of protein in which the N is degraded to urea and the S to $SO_4$, the urea and $SO_4$ accumulating as such in the body during estivation, to be rapidly excreted as complete waste products on the resumption of aquatic life; and a second category which includes creatine and $PO_4$ (and possibly some K) and which pertains to the skeletal muscles, the metabolism of these substances being either cyclical in the closed system of the estivating fish or completely suspended in consequence of the complete cessation of muscular activity during estivation. In addition no $NH_3$ formation occurs in estivating fish.

We found no more than traces of uric acid in the blood and the urine of freshly caught lung-fish (Table I). We tested the tungstate filtrates prepared from the muscles of estivating Fish 15 and 8 by Benedict's method (1922); the color developed corresponded to 0.1 mg. per cent. It is, of course, doubtful whether this color was attributable to uric acid and it seems probable that *Protopterus* completely decomposes this substance. It is worth noting that the fresh water carp excretes about 0.2 per cent of its nitrogen as uric acid, as judged by the colorimetric reaction (Smith, 1929). If this fraction of the urea N had accumulated in Fish 15 and 8 as uric acid, the quantities present would have exceeded the observed by many times. We have repeatedly examined the excreta of postestivating lung-fish for uric acid without obtaining a color significantly darker than a blank. We have obtained a positive reaction in the urine of cod (1.5 to 2.5 mg. per cent of uric acid N), carp (up to 0.63 mg. per cent), goldfish (up to 0.4 mg. per cent), puffer (2.0 mg. per cent), *Lophius* (1.5 mg. per cent) (see also Grollman, 1929), bowfin (2.6 mg. per cent), red grouper (4.5 mg. per cent), and sheepshead (1.4 mg. per cent). Uric acid occurs in the blood of teleosts (Denis, 1913–14; Marshall and Grafflin, 1928), elasmobranchs (Denis, 1922), and in small amounts in the urine of the dogfish (Denis, 1912–13). It would appear that the fishes (with the possible exception of the elasmobranchs which Przylecki (1926) claims decompose uric acid) excrete at least part of their purine N in this form. Since uric acid is probably the most toxic of all identified nitrogenous metabolites normally formed in mammals, the absence of this substance in the estivating lung-fish is particularly significant. These fish have degraded approximately 20 per cent of their body protein in a year's time and retained all the nitrogenous metabolites in consequence of complete cessation of renal activity. The state of "uremia" which prevails in them is a perfectly natural one, and it is apparently borne without injury; the degradation of uric acid to some less toxic substance may contribute in large measure to this phenomenon.

We tested both the neutralized tungstate and trichloroacetic acid filtrates of Fish 8 for acetone and acetoacetic acid by the nitroprusside and ferric chloride tests. Controls were run by adding acetone to these filtrates, in which 0.02 per cent gave a positive test. We can therefore say that the amounts of these substances present were less than this quantity. Their absence would indicate that *Protopterus* is singularly free from ketosis, since this fish had been fasted and the products of metabolism had been accumulating for 362 days, during which time it must have burned at least 1 per cent of its body weight in fat. Acetone might have been excreted by the lungs, but it is doubtful whether acetoacetic acid could be disposed of otherwise than by combustion.

A single observation showed 0.170 per cent glycogen in the skeletal muscles of Fish 8 after a total period of fasting lasting about 470 days. This is perhaps a long time record relative to the permanence of this valuable carbohydrate in the muscles of a fasted animal.

TABLE V.

*Summary of Loss of Weight, Accumulation of Urea, Etc., in Estivating Protopterus.*

| Fish No. | Days. | Initial weight. | Final weight. | Urea + $NH_3$-N. | Estivating $O_2$. |
|---|---|---|---|---|---|
| | | *gm.* | *gm.* | *gm.* | *cc. per kg per hr.* |
| 12 | 378 | 248.2 | 204.0 | 1.115 | 6.5 |
| 13 | 410 | 228.1 | 168.0 | 0.977 | 8.0 |
| 21 | 427 | 181.7 | 121.2 | 0.963 | 8.5 |
| 19 | 379 | 163.5 | 113.5 | 0.945 | 8.4 |
| 17 | 191 | 114.4 | 90.8 | 0.372 | 19.6 |
| 15 | 456 | 124.0 | 85.5 | 0.745 | 5.0 |
| 5 | 455 | 156.3 | 98.0 | 1.099 | 13.0 |
| 8 | 362 | 286.0 | 235.3 | 0.800 | |
| 7 | 364 | 204.9 | 146.9 | 1.175 | 14.0 |
| Average.. | 380 | 182.3 | 132.4 | 0.884 | |

The interesting question of water equilibrium, osmotic relations, etc., in active and estivating fish will be discussed in a subsequent paper.

## SUMMARY.

In the active lung-fish about 15 per cent of the total metabolism is attributable to muscular movements, muscular tone, circulation, and respiration; the rest is attributable to non-mechanical processes of an unknown nature. About 50 per cent of the total metabolic energy is normally furnished in the active, fasting lung-fish by protein. The degraded nitrogen is excreted about equally as urea and ammonia, together with relatively constant quantities of $SO_4$ (presumably derived from protein S), $PO_4$, K, and creatine.

During estivation the fish is rigidly imprisoned in hard mud. It passes into a state of sleep or inhibition, which is apparently of nervous origin induced by postural fixation, from which it is wakened, presumably by asphyxia, when resubmerged in water. This state of sleep is not affected by temperature, metabolic rate, mild sensory stimulation, or by thyroxine, and it may be abolished before the accumulated metabolites have been excreted from the body.

The estivating fish is entirely enveloped by a cocoon formed of dried slime except at the oral aperture through which it gets air. It lives entirely without water and exogenous fuels, and urine formation is completely suspended in an atmosphere of ordinary humidity.

During estivation the metabolic rate may sink to low levels, a considerable reduction occurring in the non-mechanical as well as the mechanical metabolism. This reduction appears to be attributable primarily to progressive emaciation.

In the estivating fish protein continues to furnish a large fraction of the metabolic energy, the protein N being almost quantitatively degraded to urea which accumulates in the body to the extent of 1 to 2 per cent of body weight in 1 year. Ammonia formation is apparently suspended. When returned to water the urea is quickly excreted as such, together with large quantities of $SO_4$. The excretion of creatine and of $PO_4$, on the other hand, begins at a low level and rises slowly to the normal, basal level; it would appear that these substances are either recatabolized, or that their anabolism is suspended, during estivation. In relation to the latter interpretation it is significant that muscular activity and muscular tone are reduced to a negligible level during estivation because of the complete immobilization of the animal.

No acetone or acetoacetic acid can be detected in estivating fish, and uric acid is apparently decomposed.

The lung-fish is a less efficient animal in prolonged fasts than man; because of a larger protein combustion the former gets only about 1.6 calories per gm. of body weight lost while fasting, as compared with the 4.0 calories obtained by the latter.

## *Methods.*

The gaseous metabolism of active and estivating fish was followed in all-glass chambers constructed from so called Hempel improved desiccators with tubulature and ground glass stop-cock. An additional 3-way capillary stop-cock was added to the tubulature close to the desiccator to permit removal of gas samples.

0.2 N $CO_2$-free NaOH was placed in the upper part of the desiccator to absorb $CO_2$. The fish was kept in 100 to 500 cc. of distilled water in the bottom chamber. To insure against leaks all ground glass joints were sealed externally with paraffin in addition to the light lubricating grease ordinarily used. Analyses of the respired gases were made on about 10 cc. samples, with a specially calibrated Haldane analyzer, the samples being removed through flexible 1 mm. copper tubing with expanded brass nipples at each end to permit attachment to the chamber and the Haldane analyzer. After the respired gas was analyzed, the chamber was opened and the alkali and water removed. These were analyzed for $CO_2$ by Van Slyke manometric analysis, and nitrogenous and other constituents were determined in the water. The $O_2$ content of the water was not determined since only a negligible error is introduced by neglecting this term.

When preparing the chamber for a metabolic run, the stop-cocks were left open for 10 minutes to permit equalization of temperature, pressure, and saturation with water vapor. After recording the external barometric pressure and the temperature as shown by a thermometer within the chamber the stop-cock was closed. At the end of the run the chamber was connected by capillary tubing to a mercury manometer and the internal pressure noted. The actual volume of gas within the chamber was determined by the difference between the total volume and the volume of the alkali, water, and fish. From these data the volume of $O_2$ corrected to normal temperature and pressure could be calculated for the beginning and the end of the run and the $O_2$ consumption determined without reference to the absorbed $CO_2$. Nearly all metabolic runs were for approximately 24 hour periods, and were terminated at the time when the gas was analyzed and the water and alkali replenished. Total nitrogen was determined by Koch and McMeekin's method (1924) and direct Nesslerization; 1 cc. of 50 per cent $H_2SO_4$ solution and 3 drops of 1 per cent

$CuSO_4$ solution were used for ashing, and 12 to 13 cc. of Nessler's solution (Folin and Wu, 1919) added to the diluted residue to take care of the excess acid, the mixture being made up to 50 cc. It may be worth reiterating Folin's precautions that it is very important to maintain the full alkalinity of the Nessler's solution, and to add this to the well diluted residue quickly and uniformly but with vigorous shaking to prevent precipitation. Ammonia was determined in the water by direct Nesslerization of 5 to 20 cc. samples, with 5 cc. of Nessler's solution, the mixture being made up to 50 cc. Urea + $NH_3$-N was determined by decomposition with urease and direct Nesslerization; the samples should contain not over 0.2 mg. of urea + $NH_3$-N; otherwise precipitation occurs on the suspended particulate matter from the urease. These analyses were made within a few hours after the end of the metabolic run to prevent bacterial decomposition. The remaining water was filtered and preserved with chloroform. When six to eight batches had accumulated, measured quantities were concentrated on the hot plate and diluted to one-tenth of their original volume.

From 5 to 15 cc. of these concentrated solutions were used in the following analyses, according to the quantities of the particular constituent present. Total creatinine N was determined by heating in the water bath for 3 hours with an equal volume of N HCl, the subsequent addition of an equivalent amount of NaOH, and colorimetric determination by the Jaffe reaction with a creatinine standard (Folin, 1922). The picric acid was recrystallized three times from water and simultaneous blank determinations were made in all cases. Tests for uric acid were made by Benedict's method (1922). Phosphate was determined in the earlier work by Briggs' modification of the Bell-Doisy method (1924) and in the later work by Fiske and Subbarow's method (1925). $SO_4$ was determined by the benzidine method of Fiske (1921) after acidifying to a yellow color with bromocresol purple. For the determination of K an aliquot was ashed with $HNO_3$, $H_2SO_4$, and superoxol, fired, and dissolved in water; the K was precipitated as the cobalti-nitrite (Kramer and Tisdall, 1921), at least 4 hours and usually 18 hours being allowed for complete precipitation. The cobalti-nitrite was measured by diazotization (Briggs, 1923), 30 minutes being allowed for color development. Standard K solutions were included in each set of determinations.

The skeletal muscles of the estivating fish, No. 8, were rapidly dissected, frozen with liquid air, and triturated in a cold mortar. The tissues of Fish 5 and 15 were triturated with pulverized glass without freezing. These fish were analyzed shortly after death in the metabolism chamber. Filtrates were prepared by adding one weight of tissue to 3 volumes of water and 1 volume of 20 per cent trichloroacetic acid, or to 7 volumes of water, 1 volume of 10 per cent sodium tungstate, and 1 volume of 0.66 N $H_2SO_4$. The mixtures were allowed to stand overnight before filtration. Total creatinine was determined in the tissues of Fish 8 by the method of Rose, Helmer, and Chanutin (1927) and checked on the filtrates. On the other tissues total creatinine was determined only in the filtrates, a wholly reliable procedure because of the ease of dissolution of fish tissues. For the glycogen determination we are indebted to Dr. Kenneth Blanchard. Other constituents were determined in the tissue filtrates by the methods described for the analysis of the water from the metabolism chamber, with appropriate modifications for concentration, etc.

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# THE ESTIMATION OF BROMIDES IN BIOLOGICAL MATERIAL.*

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The problem of estimating bromides in the presence of relatively large amounts of chlorides has attracted much attention, but none of the more simple of the published methods, summaries of which are to be found in papers by Bernhardt and Ucko (1) and by Tomicek and Jansky (2), has given satisfactory results in our hands. We have made no attempt to employ potentiometric measurements, which, to be successful, require the preliminary removal of the major portion of the chlorides (3).

We have found that bromides can be almost selectively oxidized by permanganate in phosphoric acid solution, and that the resulting bromine can be quantitatively transferred to carbon tetrachloride. In the presence of much chloride a small proportion of chlorine is also taken up by the carbon tetrachloride; the separation can, however, be made practically complete by reducing the liberated halogens with sodium sulfite and repeating the process two or three times. Under the conditions adopted, any iodide which may originally have been present is oxidized to iodate and thus escapes extraction by the organic solvent.

Removal of organic matter from the original sample is of course necessary, and this involves an ashing process.[1] We have found that no appreciable loss of bromide occurs when the incineration

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[1] Lucas (4) has described a process in which this operation is avoided by precipitation and subsequent decomposition of silver halides, but the overall accuracy of the method is low (90 per cent with 10 mg., 96 per cent with 100 mg. of bromide).

is conducted in platinum vessels at a temperature of 460–475°, in presence of potassium hydroxide, as recommended by Pincussen and Roman (5). The use of nickel vessels for this purpose leads not only to notable losses, especially in the case of blood, but also to profound corrosion of the metal.

The method as finally adopted is as follows: 10 to 15 cc. of blood or 50 to 100 cc. of urine are treated with 2 cc. of 50 per cent potassium hydroxide solution and evaporated to dryness in a platinum dish (6 to 7 cm. diameter) on the steam bath. The residue is heated over a very small flame until effervescence ceases, when the dish is transferred to an electric muffle[2] and heated for 3 to 4 hours at 460–475°. When cold, the residue is extracted with 50 to 75 cc. of distilled water in several portions; the resulting solution is filtered into a platinum dish and evaporated to dryness on the steam bath. The residue is again heated at 460–475° in the muffle for 30 to 45 minutes, and dissolved in 10 to 15 cc. of water; the solution is made up to 25 cc.

A 20 cc. aliquot of this solution is placed in a 60 cc. cylindrical separatory funnel[3] and treated with 5 cc. of 85 per cent syrupy phosphoric acid. To this mixture is added enough of 0.1 N solution of potassium permanganate in dilute (1:4 by volume) phosphoric acid to bring the color of the whole equal in intensity to that of 0.02 N permanganate After standing at room temperature for 10 minutes (to insure oxidation of iodine to iodate), during which time further quantities of permanganate solution are added as necessary to maintain the color intensity, the solution is repeatedly shaken with 5 cc. quantities of pure carbon tetrachloride until three successive shakings yield perfectly colorless extracts. Addition of further quantities of permanganate may be necessary during this extraction. Losses of halogen from the stem of the funnel are minimized by allowing 1 to 2 cc. of fresh carbon tetrachloride to flow through the apparatus (without shaking) after each extraction.

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[2] It is advantageous to remove the pyrometer plug from the back of the muffle, in order to permit the access of sufficient air for complete combustion.

[3] The separatory funnels employed for this purpose must be selected with care; in some instances it has been found impossible to prevent traces of the lighter (aqueous) solution from passing through the stop-cock.

The carbon tetrachloride solutions are run directly into a second separatory funnel containing 10 cc. of 0.5 per cent sodium sulfite solution, and the whole is well shaken. After being allowed to settle, all but about 5 cc. of the tetrachloride is discarded,[4] and 2.5 cc. of 85 per cent phosphoric acid are added, followed by enough of the permanganate solution to make the color equal to that of 0.02 N. Extraction with carbon tetrachloride is carried out exactly as before, and the extracts are run into a third separatory funnel containing 10 cc. of 0.5 per cent sodium sulfite solution The entire process is repeated, and the extracts from the third oxidation are added to 20 to 25 cc. of a freshly prepared solution of 0.5 to 1.0 gm. of potassium iodide. The liberated iodine is titrated with 0.01 N thiosulfate.

TABLE I.

*Recovery of Bromine.*

| Extraction No. | No reduction. | One reduction. | Two reductions. |
|---|---|---|---|
| | *mg.* | *mg.* | *mg.* |
| 1 | 7.59 | 7.64 | 7.48 |
| 2 | 0.48 | 0.45 | 0.49 |
| 3 | 0.07 | 0.06 | 0.11 |
| 4 | 0.02 | 0.00 | 0.01 |
| 5 | 0.01 | | |
| 6 | 0.00 | | |
| Total............ | 8.17 | 8.15 | 8.09 |

### *Recovery of Bromine.*

The efficiency of the extraction procedure and completeness of the recovery of bromine on repeated oxidation and reduction cycles are indicated by the experiment shown in Table I in which 8.15 mg. quantities of sodium bromide (without chloride) were taken. With two reduction stages, solutions containing 16.30 mg. of sodium bromide gave 15.88 and 15.94 mg. of NaBr, while the recovery from 4.075 mg. was 4.09 and 4.03 mg.

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[4] This solvent may be employed without further treatment for subsequent extractions in the same analysis.

### *Removal of Chloride.*

The experiment in Table II, with 1.0 gm. quantities of sodium chloride (without bromide), shows the extent to which chlorine passes into the extract at each stage, the results being expressed in the number of cc. of 0.00962 N thiosulfate.

The final value of 0.03 cc. corresponds to 0.03 mg. of sodium bromide.

TABLE II.

*Liberation of Chlorine.*

| Extraction No. | No reduction. | One reduction. | Two reductions. | Three reductions. |
|---|---|---|---|---|
| | *cc.* | *cc* | *cc.* | *cc.* |
| 1 | 4.02 | 1.21 | 0.07 | 0.01 |
| 2 | 10.53 | 0.91 | 0.01 | 0.01 |
| 3 | 11.91 | 0.73 | 0.00 | 0.01 |
| 4 | 11.02 | 0.17 | 0.00 | 0.00 |
| Total....... | 37.48 | 3.02 | 0.08 | 0.03 |

TABLE III.

*Estimation of Bromide.*

| NaBr taken. | No. of estimations. | NaBr found | | |
|---|---|---|---|---|
| | | Minimum. | Maximum. | Average |
| *mg.* | | *mg.* | *mg.* | *mg.* |
| 50.8 | 2 | 49.5 | 49.8 | 49.7 |
| 20.32 | 2 | 19.58 | 19.75 | 19.67 |
| 16.28 | 1 | | | 15.60 |
| 10.16 | 9 | 9.33 | 10.67 | 10.07 |
| 8.14 | 2 | 7.77 | 7.82 | 7.80 |
| 5.08 | 2 | 5.06 | 5.07 | 5.06 |
| 4.07 | 2 | 3.84 | 3.90 | 3.87 |
| 1.628 | 3 | 1.63 | 1.69 | 1.66 |
| 1.016 | 2 | 1.11 | 1.19 | 1.15 |
| 0.814 | 2 | 0.84 | 0.89 | 0.87 |
| 0.508 | 2 | 0.64 | 0.70 | 0.67 |

### *Variation in Amount of Bromide.*

Determinations were run with various quantities of sodium bromide in the presence, in each case, of 1.0 gm. of sodium chloride, two reduction stages being employed. The results are shown in Table III.

### *Influence of Iodide.*

A mixture of 1 gm. of NaCl, 4.07 mg. of NaBr, and 6.015 mg. of KI in 20 cc. was analyzed, two reduction stages being employed, with four extractions after each oxidation. Found 4.12, 4.02 mg. of NaBr.

### *Loss of Bromide on Incineration.*

Two composite samples of human blood were analyzed, with and without the addition of 9.90 mg. quantities of sodium bromide to each 12 cc portion taken for analysis. The procedure described above was followed exactly in duplicate, two reduction stages being employed.

| Sample. | Without added NaBr. | | Average. | With 9.90 mg. added NaBr. | | Average. | Average difference. |
|---|---|---|---|---|---|---|---|
| | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| A | 1.25 | 1.74 | 1.50 | 11.03 | 11.60 | 11.32 | 9.82 |
| B | 0.72 | 0.94 | 0.83 | 10.28 | 10.90 | 10.59 | 9.76 |

The loss is thus within the error of the analytical procedure.

## SUMMARY.

A simple and moderately accurate method is described for the estimation of bromides in biological fluids, based on the selective oxidation of bromide by permanganate in dilute phosphoric acid.

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# THE PARTIAL DEHYDROGENATION OF α- AND β-AMYRIN.

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In recent years polyterpene chemistry has been greatly advanced, especially through the work of Ruzicka and his coworkers, by the application of the method of exhaustive dehydrogenation with sulfur, and more recently by the use of the selenium method of Diels.[1] During the past year Ruzicka has extended the study to the more complex triterpene derivatives, such as the amyrins[2] and various sapogenins.[3] Although frequent reference has been made to the probable close relationship of the sapogenins, sterins, and other higher terpenes, it is very necessary to continue the study in order to determine the real particulars of these relationships. In a report of their experiments on the amyrins Ruzicka, Huyser, Pfeiffer, and Seidel[2] obtained by dehydrogenation with sulfur[4] and preferably with selenium a hydrocarbon, $C_{13}H_{14}$, which was isolated as the picrate or styphnate and was found to be a trimethylnaphthalene. This hydrocarbon on oxidation gave a naphthalenetricarbonic acid. Since in a subsequent work Ruzicka and van Veen[3] succeeded in obtaining the same hydrocarbon by dehydrogenation with selenium of a series of sapogenins and sterins, although with certain exceptions, they have proposed the name

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[1] Diels, O., and Karstens, A., *Ber. chem. Ges.*, **60**, 2323 (1927). Diels, O., Gädke, W., and Körding, P., *Ann. Chem.*, **459**, 1 (1927).

[2] Ruzicka, L., Huyser, H. W., Pfeiffer, M., and Seidel, C. F., *Ann. Chem.*, **471**, 21 (1929).

[3] Ruzicka, L., and van Veen, A. G., *Z. physiol. Chem.*, **184**, 69 (1929); *Rec. trav. chim. Pays-Bas*, **48**, 1018 (1929).

[4] Brunner, O., (*Monatsh. Chem.*, **50**, 287 (1928)) has also recently reported experiments on the dehydrogenation of the mixed amyrins with sulfur which, however, were very inconclusive.

sapotalin for this trimethylnaphthalene. Although the mechanism of the formation of this substance is still unknown, they have been able to conclude that there is a close relationship between a number of these sapogenins and natural triterpene derivatives. This conclusion in so far as β-amyrin and hederagenin are concerned has been extended by our own work which is presented in the present and following communications.

Before the appearance of the experiments of Ruzicka and coworkers on the dehydrogenation of amyrin, we had already in progress a study of the dehydrogenation of this substance. Since our point of view and immediate objective differed from those of these workers we have therefore continued these studies. In the dehydrogenation experiments already mentioned, the purpose has been exhaustive treatment in order to achieve complete conversion into aromatic hydrocarbons which might be easier of recognition. It occurred to us that it might be possible to interrupt the dehydrogenation at proper points and so obtain substances which represent partial dehydrogenation before too deep seated changes have occurred. The study of such derivatives might then prove useful in giving clues to the structure of the parent substances. With this in view, the examination of a series of substances has been begun, including the sapogenins, resin alcohols, and other polyterpene derivatives as well as the cardiac aglucones and other members of the saturated polycyclic group. Our preliminary results have demonstrated the possible usefulness of this method of approach. We shall present here the experience with the amyrins.

These experiments have been made with pure α- and β-amyrin which were prepared by the method of Vesterberg and Westerlind[5] from the crude mixture of the isomers obtained from gum elemi as well as with their benzoates. When α- and β-amyrin were heated in an atmosphere of nitrogen with an equal weight of sulfur, a slow but steady evolution of hydrogen sulfide occurred at 200–210°, which continued for several hours with the bath at this temperature, and then gradually ceased. In the case of the benzoates it was necessary to go somewhat higher, to 220–230°, in order to obtain a parallel result. If in any case the temperature was

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[5] Vesterberg, K. A., and Westerlind, S., *Ann. Chem.*, **428**, 243 (1922).

raised, as previous observations have already shown, there was of course further dehydrogenation with extensive carbonization. If, however, the reaction was interrupted at this point, it was possible to isolate definite transformation products. This was accomplished by extraction of the reaction mixture and fractionation of the soluble material at low pressure. The resulting resins were then crystallized from appropriate solvents. But the substances obtained in the case of β-amyrin and its benzoate differed in character from those obtained from the α compounds. β-amyrin or its benzoate yielded sulfur compounds which from the formulations deduced from the analytical results, respectively $C_{30}H_{44}OS$ and $C_{37}H_{48}O_2S$, have been formed by the removal of 3 mols of hydrogen and the introduction of 1 sulfur atom. The reactions occurring in the case of β-amyrin and its benzoate were identical since the thio compounds obtained from both sources were interconvertible by benzoylation or saponification. The yield in the case of the benzoate (about 50 per cent) was somewhat better than in the case of β-amyrin itself (35 per cent). The secondary hydroxyl group of β-amyrin is, therefore, retained in the sulfur derivative.

The sulfur of the thio compounds is apparently of complex thiophene character since it cannot be removed as hydrogen sulfide on heating with strong alcoholic alkali. On the other hand, it was readily removed in oxidation experiments, as given below. Attempts to demonstrate the presence of new double bonds by catalytic hydrogenation experiments were unsuccessful. This fact, however, does not exclude the presence of double bonds.

In the oxidation experiments the benzoate of the thio compound was employed in order to avoid complications which might arise by participation in the reaction of the secondary hydroxyl group. With permanganate in acetic acid, it was gradually oxidized with the formation of at least two substances of neutral character which no longer contained sulfur. Although it was possible to separate these substances by fractional recrystallization, it was found preferable to saponify the crude benzoate mixture. From the resulting mixture of hydroxy compounds the two substances were separated by fractional recrystallization and gave analytical figures which agreed best with the formulæ $C_{30}H_{46}O_3$ and $C_{30}H_{44}O_4$ respectively.

On acylation both of these substances gave only a monoacetate or a monobenzoate, the latter proving to be identical in each case with the benzoates obtained directly in the above oxidation. Therefore, aside from the secondary hydroxyl group originally present in amyrin, no other hydroxyl group can be detected by acylation. The substance $C_{30}H_{46}O_3$ proved to be a ketone since it yielded, although with some difficulty, a monooxime. By the Clemmensen method only 1 oxygen atom could be replaced by hydrogen. The remaining oxygen atom introduced during the oxidation is, therefore, of undetermined character although it is presumably a hydroxyl. The substance resisted all attempts to cleave it on heating with strong alkali.

The substance $C_{30}H_{44}O_4$ is apparently a lactone. Although it resisted boiling in 5 per cent alcoholic alkali for hours, when heated with 10 per cent alkali at 130° it was converted into a monobasic acid, $C_{30}H_{46}O_5$. The same substance was encountered in the reaction mixture obtained on saponification of the crude benzoate mixture. The lactone did not react with hydroxylamine. It formed only a monoacetate and a monobenzoate and the latter proved to be identical with that isolated directly from the oxidation mixture. No indication of the absorption of hydrogen was obtained in experiments on catalytic hydrogenation.

The above ketone, however, was found to absorb catalytically activated hydrogen and the resulting substance appeared to be a mixture of isomers. The analytical figures, although not in close agreement, approximated those for amyrin, indicating definitely the loss of the 2 extra oxygen atoms during the hydrogenation, and since it still gave a benzoate, the retention of the original secondary hydroxyl.

Owing to the temporary lack of sufficient material we have been forced to interrupt this work for the present. With the data at hand it is perhaps premature to suggest a picture of the mechanism of the formation of these oxidation products from the thio compound and much less the relationship of the latter to the parent triterpene alcohol. Although several interpretations are possible, we shall leave this rather until further work has been done.

In the recent work of Ruzicka with Pfeiffer[6] and with van Veen[7]

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[6] Ruzicka, L., and Pfeiffer, M., *Helv. Chim. Acta*, **9**, 841 (1926).

[7] Ruzicka, L., and van Veen, A. G., *Ann. Chem.*, **476**, 70 (1929).

on the dehydrogenation of elemol with sulfur, a sulfur-containing dehydrogenation product has also been obtained for which a thionaphthalene structure has been proposed. The possibility of any structural analogy between this substance and our own must be left for the present. It might be further noted that in the dehydrogenation of colophonium oil with sulfur along with retene, a crystalline sulfur compound, $C_{18}H_{16}S$, has been described by Schultze[8] as a thioretene which was shown to be a secondary product due to the further action of sulfur on retene.

In the case of both α-amyrin and its benzoate the reaction with sulfur was found to take a different course, at least in so far as was indicated by the study of the reaction product which was obtained. The reaction with sulfur was performed exactly as in the case of the β-isomers. Much more carbonization occurred than in the case of the β-isomer and a much smaller yield, only about 10 per cent, of a crystalline substance was isolated, which was found to contain no sulfur. The analysis gave figures which agreed with the formulæ $C_{30}H_{48}O$ and $C_{37}H_{52}O_2$ for the free hydroxy compound and the benzoate respectively. This indicates that its formation from α-amyrin has resulted from the loss of 1 mol of hydrogen. Characteristic of dehydro-α-amyrin is its high rotation, $[\alpha] = +358°$, and the indigo blue color which it instantly gives with the Liebermann cholesterol test. For further characterization dehydro-α-amyrin was oxidized with chromic acid to the ketone, dehydro-α-amyrone, from which an oxime was prepared. For the time being, its behavior on oxidation with permanganate has not been studied.

The difference in the behaviors of α- and β-amyrin on partial dehydrogenation with sulfur brings up for the first time the question whether the difference in structure between these isomers is merely stereochemical. On the other hand, as will be seen in the following communication on a derivative of hederagenin, there is a striking parallelism in the behaviors of this sapogenin derivative and β-amyrin, which increases the probability of the close relationship between these substances, since analogous structural features are probably involved in each case in the dehydrogenation and cyclization with sulfur.

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[8] Schultze, W., *Ann. Chem.*, **359**, 129 (1908).

## EXPERIMENTAL.

### *Partial Dehydrogenation of β-Amyrin and Benzoate.*

In the dehydrogenation experiments it was found useful in all cases to employ a long necked 50 cc. Pyrex flask fitted with a ground glass stopper of wash bottle type containing an inlet and an exit tube. The latter was connected with a small trap which was in turn connected with a bubble counter containing about 1 cc. of water. After introduction of the mixture of substance and sulfur into the flask, the stopper joint was made gas-tight with sulfur and air was displaced from the apparatus by a stream of nitrogen. This stream was then discontinued and the flask was heated in a metal bath. The temperature was gradually raised until a slow, steady stream of $H_2S$ was evolved. The temperature was then maintained within 10° of this point as long as appreciable evolution of gas continued. The temperature and duration will be recorded in each case.

*Dehydrogenation of β-Amyrin Benzoate.*—A mixture of 9.0 gm. of β-amyrin benzoate and an equal amount of sulfur was heated for 4 hours at a bath temperature of 220–230°. The reaction mass was extracted with benzene and the excess sulfur removed by filtration. The benzene solution was evaporated to dryness on the steam bath and the resulting solid was distilled at a pressure of 2 mm. in 3 gm. portions from a 10 cc. Claisen distilling flask. Solidification of the distillate in the delivery tube of the distilling flask was prevented by inserting a copper wire through the delivery tube and extending this wire into the side neck of the Claisen flask. The temperature of the metal bath, in which the flask was immersed, was gradually increased until 275° was attained. At this point practically all of the free sulfur had distilled off. A cherry-colored resin distilled when the temperature of the bath was maintained between 300–350°. The resin was dissolved in a small amount of benzene and precipitated with 2 volumes of methyl alcohol. The precipitate was dissolved in benzene and decolorized with norit. When this solution was concentrated, the substance crystallized in the form of prisms which melted at 224–225°. The yield was 50 per cent.

$[\alpha]_D^{25} = +96°$ (c = 1.017 in pyridine).
4.596 mg. substance: 3.610 mg. $H_2O$, 13.424 mg. $CO_2$.
4.648 " " : 3.720 " " 13.600 " "
6 654 " " : 2.755 " $BaSO_4$.
$C_{37}H_{48}O_2S$. Calculated. C 79.80, H 8.70, S 5.75.
Found. (*a*) " 79.66, " 8.79.
(*b*) " 79.80, " 8.96.
(*c*) S 5.68

Molecular weight determination according to the method of Rast[9] gave the following results:

29.160 mg. camphor: 3.234 mg. substance, $\Delta = 8.75$. Mol. wt., calculated, 556.4; found, 570.

The Liebermann cholesterol test gave an amber-colored solution which changed into a reddish brown when warmed. Attempts to benzoylate the substance with pyridine and benzoyl chloride resulted in recovery of unchanged material. When this substance was refluxed for 6 hours in an acetic acid solution of lead acetate, no evidence of the cleavage of sulfur was noted and the starting material was recovered unchanged.

*Saponification of the Benzoate, $C_{37}H_{48}O_2S$.*—A solution of 0.5 gm. of the above sulfur compound in 50 cc. of 10 per cent alcoholic KOH was refluxed for 5 hours. The product was precipitated from the boiling solution by addition of water. When recrystallized from alcohol it separated in the form of fine needles which melted at 200–201°.

$[\alpha]_D^{30} = +88°$ (c = 1.017 in pyridine).
6.105 mg. substance: 5.335 mg. $H_2O$, 17.840 mg. $CO_2$.
6.788 " " : 5.940 " " 19.785 " "
$C_{30}H_{44}OS$. Calculated. C 79.60, H 9.82.
Found. (*a*) " 79.70, " 9.78.
(*b*) " 79.50, " 9.79.

The cholesterol test gave a cherry-red color which gave way to a yellowish green when the solution was warmed.

*Dehydrogenation of β-Amyrin.*—A mixture of 1.0 gm. of β-amyrin and an equal amount of sulfur was heated for 3 hours at a bath temperature of 200–210°. The reaction mass was extracted with

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[9] Smith, J. H. C., and Young, W. G., *J. Biol. Chem.*, **75**, 289 (1927).

ether and the ether solution was concentrated to a small volume and then filtered into a 10 cc. Claisen distilling flask. The residue was distilled at 2 mm. with the bath between 300–350°. The cherry-colored resin obtained in this manner was taken up in alcohol and the solution was decolorized with charcoal. The substance was precipitated from a boiling hot solution by addition of water. When this product was recrystallized from alcohol it separated in the form of fine needles which melted at 200–201°. The yield was 35 per cent.

$[\alpha]_D^{25}$ = +86° (c = 1.020 in pyridine).
4.805 mg. substance: 4.222 mg. $H_2O$, 14.050 mg. $CO_2$.
3.665 " " : 3.243 " " 10.640 " "
7.432 " " : 3.885 " $BaSO_4$.
$C_{30}H_{44}OS$. Calculated. C 79.60, H 9.82, S 7.08.
Found. (*a*) " 79.74, " 9.83.
(*b*) " 79.17, " 9.90.
(*c*) S 7.18.

The cholesterol test gave a cherry-red color which on heating gave way to a yellowish green. No depression of melting point could be observed when this substance was mixed with that obtained by saponification of the above benzoate. The identity of the substances obtained was confirmed by comparison of their benzoates.

A solution of 0.1 gm. of $C_{30}H_{44}OS$, obtained from β-amyrin, in 1 cc. of benzene was refluxed with an excess of pyridine and benzoyl chloride for 1 hour. After evaporation on the steam bath, the residue was recrystallized from benzene by addition of methyl alcohol. The substance melted at 224–225° and showed no depression when mixed with the previous benzoate. In all other properties it proved to be identical.

$[\alpha]_D^{25}$ = +97° (c = 1.000 in pyridine).
4.040 mg. substance: 3.210 mg. $H_2O$, 11.820 mg. $CO_2$.
4.542 " " : 3.558 " " 13.330 " "
10.215 " " : 3.960 " $BaSO_4$.
$C_{37}H_{48}O_2S$. Calculated. C 79.80, H 8.70, S 5.75.
Found. (*a*) " 79.75, " 8.89.
(*b*) " 80.00, " 8.77.
(*c*) S 5.33.

### *Oxidation of the Benzoate, $C_{37}H_{48}O_2S$.*

A suspension of 5 gm. of the benzoate in 500 cc. of acetic acid was stirred vigorously while 190 cc. of 6 per cent $KMnO_4$ solution was added. After stirring for 2 hours complete solution occurred. This solution was then diluted with 1 liter of water and the precipitate was separated by filtration from the colloidal solution of $MnO_2$. 4.5 gm. of a mixture of benzoates were obtained, which was found to consist principally of the esters of the ketone $C_{30}H_{46}O_3$ and the lactone $C_{30}H_{44}O_4$. Although the separation of these substances was accomplished directly by fractionation from ligroin, it was found to be more advantageous to saponify the benzoates. For this purpose the crude substance was refluxed in 100 cc. of 10 per cent alcoholic KOH for 4 hours. After dilution, the resulting neutral precipitate was collected. The yield was 2.45 gm. When the alkaline filtrate was acidified, 1.1 gm. of an acid fraction was obtained. From the neutral fraction the ketone and lactone were separated as follows.

*The Lactone, $C_{30}H_{44}O_4$.*—Repeated recrystallizations from methyl alcohol of the above neutral fraction yielded homogeneous stout prisms which melted constantly at 299–300° with decomposition and were readily soluble in the usual solvents. Although this substance was recovered unchanged after prolonged heating at 130° with 5 per cent alcoholic KOH, more vigorous treatment as given below causes saponification to the acid, thus indicating its lactone character. It is not affected by concentrated hydrochloric acid at ordinary temperatures. The cholesterol test gave a reddish brown color which deepened when warmed.

$[\alpha]_D^{30} = -14°$ (c = 1.010 in pyridine).

4.540 mg. substance: 3.880 mg. $H_2O$, 12.855 mg. $CO_2$.
4.810 " " : 4.100 " " 13.570 " "

| | | | | |
|---|---|---|---|---|
| $C_{30}H_{44}O_4$. | Calculated. | | C 76.90, | H 9.48. |
| | Found. | (a) | " 77.22, | " 9.57. |
| | | (b) | " 76.94, | " 9.54. |

*The Acetate of the Lactone, $C_{30}H_{44}O_4$.*—A solution of 0.05 gm. of the lactone and 0.05 gm. of fused sodium acetate in 2 cc. of acetic anhydride was refluxed for 7 hours. At the end of this period the solution was diluted with water and the collected precipitate was recrystallized from dilute alcohol. It crystallized in the form of shining plates which melted at 269–271°.

5.048 mg. substance: 4.105 mg. $H_2O$, 13.930 mg. $CO_2$.
4.600 " " : 3.670 " " 12.645 " "
$C_{32}H_{46}O_5$. Calculated. C 75.41, H 9.09.
Found. (*a*) " 75.26, " 9.10.
(*b*) " 74.97, " 8.93.

*The Benzoate of the Lactone, $C_{30}H_{44}O_4$.*—A solution of 0.05 gm. of the lactone in benzene was refluxed for 3 hours with an excess of pyridine and benzoyl chloride. The excess of reagents was removed by repeated evaporation with benzene. The residue after recrystallization from alcohol formed needles which melted at 292–293°.

3.917 mg. substance: 2.940 mg. $H_2O$, 11.178 mg. $CO_2$.
3.845 " " : 3.005 " " 10.930 " "
$C_{37}H_{48}O_5$. Calculated. C 77.60, H 8.47.
Found. (*a*) " 77.83, " 8.40.
(*b*) " 77.52, " 8.74.

The same benzoate was obtained directly by fractionation from ligroin of the crude mixture resulting from the oxidation of the benzoate of the thio compound. After repeated recrystallization from alcohol it formed needles which melted at 295–297° and showed no depression when mixed with the above benzoate.

3.992 mg. substance: 3.035 mg. $H_2O$, 11.312 mg. $CO_2$.
4.738 " " : 3.654 " " 13.485 " "
Found. (*a*) C 77.27, H 8.51.
(*b*) " 77.62, " 8.63.

*The Acid, $C_{30}H_{46}O_5$.*—A solution of 0.1 gm. of the lactone in 6 cc. of 10 per cent alcoholic KOH was heated in a sealed tube at 120–130° for 3 hours. The diluted mixture was acidified and the precipitate of acid and silica was collected and dried. The alcoholic solution was filtered from silica. On dilution of the filtrate, the crystalline acid separated as prisms. After recrystallization from acetone it melted at 237–238°.

4.055 mg. substance: 3.480 mg. $H_2O$, 11.030 mg. $CO_2$.
$C_{30}H_{46}O_5$. Calculated. C 74.01, H 9.54.
Found. " 74.19, " 9.60.

For the titration 12.702 mg. of the acid were dissolved in 2 cc. of alcohol and titrated with 0.1 N NaOH against phenolphthalein. Found, 0.276 cc. Calculated for 1 equivalent, 0.261 cc. After refluxing with an excess of alkali no further consumption was noted.

The same acid was obtained directly from the crude acid fraction formed on the saponification of the original oxidation mixture. This crude fraction on recrystallization from alcohol formed prisms which after being recrystallized from acetone melted at 238–239°.

$[\alpha]_D^{23} = +96°$ (c = 1.030 in pyridine).
3.912 mg. substance: 3.330 mg. $H_2O$, 10.580 mg. $CO_2$.
4.777 " " : 4.075 " " 12.922 " "
$C_{30}H_{46}O_5$. Calculated. C 74.01, H 9.54.
Found. (a) " 73.76, " 9.53.
(b) " 73.78, " 9.55.

8.575 mg. of substance were dissolved in 2 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found, 0.200 cc. Calculated for 1 equivalent, 0.177 cc.

*The Ketone, $C_{30}H_{46}O_3$.*—This substance was isolated from the crude methyl alcoholic mother liquors obtained from the above lactone. After repeated recrystallization from ether it formed long slender needles which melted at 274–275°.

$[\alpha]_D^{26} = -127°$ (c = 1.023 in pyridine).
4.285 mg. substance: 3.998 mg. $H_2O$, 12.455 mg. $CO_2$.
4.700 " " : 4.264 " " 13.670 " "
$C_{30}H_{46}O_3$. Calculated. C 79.30, H 10.22.
Found. (a) " 79.27, " 10.43.
(b) " 79.32, " 10.15.

This substance may be recovered from its solution in concentrated hydrochloric acid unchanged. The cholesterol test gave a yellow-orange color which changed to amber when the solution was warmed.

*The Oxime of the Ketone, $C_{30}H_{46}O_3$.*—A solution of 0.1 gm. of the ketone in alcohol was refluxed for 5 hours with twice the calculated amounts of sodium acetate and hydroxylamine hydrochloride. The solution on dilution with water gave a precipitate which was recrystallized from acetone. It separated in the form of prisms which melted at 234–236° with decomposition.

3.820 mg. substance: 3.385 mg. $H_2O$, 10.740 mg. $CO_2$.
7.557 " " : 0.220 cc. N (29°, 756.0 mm.).
$C_{30}H_{47}O_3N$. Calculated. C 76.69, H 10.08, N 3.00.
Found. (*a*) " 76.70, " 9.92.
(*b*) N 3.27.

*The Acetate of the Ketone, $C_{30}H_{46}O_3$.*—A solution of equal amounts of the ketone and fused sodium acetate in acetic anhydride was refluxed for 7 hours. The solution was diluted with water and the resulting substance was recrystallized from alcohol by dilution with water. The melting point observed was 231–232°. The low hydrogen figures obtained on analysis of this substance were not further investigated.

4.605 mg. substance: 3.908 mg. $H_2O$, 13.045 mg. $CO_2$.
4.390 " " : 3.670 " " 12.403 " "
$C_{32}H_{48}O_4$. Calculated. C 77.36, H 9.75.
Found. (*a*) " 77.25, " 9.50.
(*b*) " 77.06, " 9.35.

*The Benzoate of the Ketone, $C_{30}H_{46}O_3$.*—A benzene solution of 0.2 gm. of the ketone was refluxed for 1 hour with an excess of pyridine and benzoyl chloride. After evaporation, the residue was taken up in alcohol and precipitated with water. The benzoate separated as needles when recrystallized from alcohol and melted at 261–262°.

$[\alpha]_D^{24} = -77°$ (c = 1.003 in pyridine).
2.920 mg. substance: 2.365 mg. $H_2O$, 8.518 mg. $CO_2$.
4.625 " " : 3.657 " " 13.478 " "
$C_{37}H_{50}O_4$. Calculated. C 79.51, H 9.02.
Found. (*a*) " 79.56, " 9.06.
(*b*) " 79.48, " 8.85.

This benzoate proved to be resistant to the oxidizing action of permanganate in acetic acid solution. Apparently the same benzoate, although not quite pure, was obtained directly from the mother liquor after removal of the benzoate of the lactone from the mixture of crude benzoates which resulted from the oxidation of the sulfur compound. Repeated recrystallization from alcohol finally yielded needles which melted at 255–257°, somewhat lower than the above benzoate. When mixed no further depression of the melting point was observed.

4 109 mg. substance: 3.153 mg. $H_2O$, 11.897 mg. $CO_2$.
4.035 " " : 3.200 " " 11.660 " "
Found. (*a*) C 78.98, H 8.59.
(*b*) " 78.81, " 8.88.

*Clemmensen Reduction of the Ketone, $C_{30}H_{46}O_3$.*—A solution of 0.05 gm. of the ketone in 5 cc. of acetic acid was treated with 2 gm. of amalgamated zinc and 1 cc. of hydrochloric acid (1.19). The mixture was refluxed for 1½ hours and then precipitated with water. When recrystallized from dilute alcohol, the desoxy derivative melted not sharply at 155–160°. It is to be doubted that the substance was homogeneous and owing to the limited amount of material available it was not investigated further.

4.252 mg. substance: 4.135 mg. $H_2O$, 12.610 mg. $CO_2$.
4.480 " " : 4.460 " " 13.267 " "
$C_{30}H_{48}O_2$. Calculated. C 81.75, H 10.98.
Found. (*a*) " 80.89, " 10.88.
(*b*) " 80.77, " 11.14.

*Hydrogenation of the Ketone, $C_{30}H_{46}O_3$.*—0.2 gm. of the ketone in alcoholic solution was hydrogenated with 0.1 gm. of platinum oxide catalyst. After reduction of the catalyst the hydrogen absorption proceeded slowly but steadily. Since after 18 hours the addition of more catalyst caused no further absorption, the operation was discontinued. The volume of 40 cc. noted was approximately the equivalent of 4 mols of hydrogen. On concentration after removal of the catalyst, the substance crystallized as needles which melted at 205–215°. This material was probably a mixture of isomers and after repeated recrystallization from alcohol it melted at 200–202°. The analytical figures approximated those for amyrin.

3.700 mg. substance: 3.745 mg. $H_2O$, 11.405 mg. $CO_2$.
3.845 " " : 3.835 " " 11.815 " "
3.933 " " : 4.000 " " 12.080 " "
$C_{30}H_{50}O$. Calculated. C 84.45, H 11.73.
Found. (*a*) " 84.07, " 11.33.
(*b*) " 83.81, " 11.17.
(*c*) " 83.77, " 11.38.

In a second hydrogenation experiment a similar substance was obtained which on repeated recrystallization from methyl alcohol melted not sharply from 207–226° and was apparently a mixture of stereoisomers.

4.437 mg. substance: 4.425 mg. $H_2O$, 13.620 mg. $CO_2$.
Found. C 83.72, H 11.16.

This substance did not react with hydroxylamine but yielded a benzoate. This proved to be a mixture of isomers which melted from 205–215°. The analytical figures, however, with several samples of repeatedly recrystallized benzoate gave analytical figures which were in consistent disagreement with the calculated figures for a monobenzoate of $C_{30}H_{50}O$. The following is representative of these results.

4.258 mg. substance: 3.628 mg. $H_2O$, 12.950 mg. $CO_2$.
$C_{37}H_{54}O_2$. Calculated. C 83.71, H 10.26.
Found. " 82.94, " 9.54.

### *Partial Dehydrogenation of α-Amyrin and Benzoate.*

*Dehydrogenation of α-Amyrin Benzoate.*—A mixture of 6.0 gm. of α-amyrin benzoate and an equal amount of sulfur was heated in an atmosphere of nitrogen for 6 hours at a bath temperature of 220–230°. Considerable carbonization occurred. The reaction mass was extracted with ether and the filtered solution was evaporated to dryness. When ether was again added to the residue complete solution did not take place. This undissolved sulfur was removed by filtration and the solution was again evaporated to dryness. This process was repeated until addition of ether produced complete solution. The final ether-soluble product from this treatment was dissolved in alcohol and the solution was decolorized with carbon. On concentration, dehydro-α-amyrin benzoate crystallized in the form of shining plates. The product was recrystallized from alcohol and then from acetone. 0.6 gm. of substance was obtained which melted at 171–172°. The cholesterol test proved to be quite characteristic and of a different character from that shown by α- and β-amyrin benzoates. This benzoate gave a light blue color which deepened to an indigo blue when the solution was warmed for a short time.

$[\alpha]_D^{23}$ = +304° (c = 1.023 in pyridine).
5.237 mg. substance: 4.600 mg. $H_2O$, 16.115 mg. $CO_2$.
4.175 " " : 3.718 " " 12.813 " "
$C_{37}H_{52}O_2$. Calculated. C 84.03, H 9.92.
Found. (*a*) " 83.92, " 9.83.
(*b*) " 84.10, " 10.00.

*Dehydro-α-Amyrin Benzoate from α-Amyrin.*—A mixture of 1.7 gm. of α-amyrin and an equal amount of sulfur was heated in an atmosphere of nitrogen for 5 hours at a bath temperature of 200–210°. The mass was extracted with ether and the sulfur removed as in the case of the above benzoate. It was found advantageous to convert this product into the benzoate for purposes of purification. Accordingly, it was dissolved in benzene and was refluxed for 1 hour with benzoyl chloride and pyridine. The excess reagents were removed by repeated evaporation with benzene. The resulting syrup was taken up in alcohol and after treatment with norit the solution was concentrated on the steam bath until crystallization took place. The resulting substance was identical with the dehydro-α-amyrin benzoate described above. It melted at 171–172°.

$[\alpha]_D^{23}$ = +305° (c = 1.007 in pyridine).
4 902 mg. substance: 4.370 mg. $H_2O$, 15.110 mg. $CO_2$.
$C_{37}H_{52}O_2$. Calculated. C 84.03, H 9.92.
Found. " 84.06, " 9.98.

*Dehydro-α-Amyrin.*—The benzoate was refluxed with 10 parts of 10 per cent alcoholic KOH for 5 hours. The hot solution was diluted with water. The resulting precipitate was collected with water and recrystallized from hot acetone by dilution with water. It crystallized in the form of long, slender needles which melted at 162–163°. The cholesterol test gave an immediate deep indigo-blue in the cold.

$[\alpha]_D^{29}$ = +358° (c = 1.000 in pyridine).
4.202 mg. substance: 4.278 mg. $H_2O$, 13.085 mg. $CO_2$.
3.977 " " : 3.950 " " 12.385 " "
$C_{30}H_{48}O$. Calculated. C 84.91, H 11.32.
Found. (*a*) " 84.92, " 11.39.
(*b*) " 84.93, " 11.12.

*Dehydro-α-Amyrone.*—A solution of 0.2 gm. of dehydro-α-amyrin in 5 cc. of acetic acid was oxidized by slow addition of an acetic acid solution of 0.07 gm. of $CrO_3$. This solution was refluxed for ½ hour and then diluted while hot. The resulting substance when recrystallized from methyl alcohol separated in the form of plates which melted at 133–134°. The cholesterol test gave a light violet color which deepened when the solution was warmed and then gradually changed to a green.

$[\alpha]_D^{20} = +412°$ (c = 0.870 in pyridine).
3.964 mg. substance: 3.860 mg. $H_2O$, 12.310 mg. $CO_2$.
4.450 " " : 4.402 " " 13.833 " "
4.125 " " : 4 138 " " 12.915 " "
$C_{30}H_{46}O$. Calculated. C 85.20, H 10.73.
Found. (a) " 84.70, " 10.90.
(b) " 84.78, " 11.07.
(c) " 85.39, " 11.22.

*α-Amyrone.*—For comparison α-amyrone was prepared according to Zinke.[10] It possessed the recorded properties and melted at 124° after preliminary sintering. The cholesterol test gave a light violet color which gradually faded to a yellow when warmed.

$[\alpha]_D^{25} = +119°$ (c = 1.007 in pyridine).
4.558 mg. substance: 4.572 mg. $H_2O$, 14.185 mg. $CO_2$.
4.733 " " : 4.833 " " 14.690 " "
$C_{30}H_{48}O$. Calculated. C 84.91, H 11.32.
Found. (a) " 84.87, " 11.23.
(b) " 84.65, " 11.42.

*Dehydro-α-Amyroneoxime.*—A methyl alcoholic solution of 0.05 gm. of dehydro-α-amyrone was refluxed with an excess of hydroxylamine hydrochloride and sodium acetate for 2 hours. The oxime was precipitated from the boiling solution by the addition of water. When recrystallized from methyl alcohol it separated in the form of needles which melted at 233–235°.

4.332 mg. substance 4.200 mg. $H_2O$, 13.120 mg. $CO_2$.
2.230 " " 2.110 " " 6.743 " "
4.728 " " 0.136 cc. N (26°, 775.9 mm.).
$C_{30}H_{47}ON$. Calculated. C 82.30, H 10.84, N 3.20.
Found. (a) " 82.60, " 10.85.
(b) " 82.47, " 10.60.
(c) N 3.35.

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[10] Zinke, A., *Monatsh. Chem.*, **42**, 439 (1924).

# SAPONINS.

## V. THE PARTIAL DEHYDROGENATION OF HEDERAGENIN.

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Simultaneously with the experiments on the partial dehydrogenation with sulfur of the amyrins,[1] we have also carried on analogous studies with hederagenin derivatives. In preliminary experiments with hederagenin methyl ester a relatively small amount of a crystalline sulfur compound was obtained, but since much better results were secured with another derivative the investigation of this substance was discontinued. With the idea that the free hydroxyl groups of hederagenin methyl ester contributed perhaps to the very poor yield of sulfur derivative, we have turned to another substance in which these groups have been removed. The derivative selected was the previously described substance $C_{31}H_{50}O_2$,[2] which was prepared by the Clemmensen reduction of the ketone, $C_{31}H_{48}O_3$. This ketone had been in turn obtained by oxidative degradation of hederagenin methyl ester. For convenience of designation we propose the name hedragonic methyl ester for this ketone and for its reduction product, $C_{31}H_{50}O_2$, the name hedraganic methyl ester. When the latter substance was partially dehydrogenated with sulfur, about 50 per cent of a crystalline sulfur-containing derivative was obtained in which the methyl ester group remained intact. The formula of this substance suggested by the analytical figures is $C_{31}H_{44}O_2S$ which must have been formed by the removal of 3 mols of hydrogen and the introduction of 1 sulfur atom. This derivative proved to be analogous to the sulfur compound obtained from β-amyrin

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[1] Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **88**, 137 (1930).

[2] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **69**, 641 (1926).

The sulfur atom resisted treatment with alkali and is unquestionably of cyclic character. The analogy of this substance to the β-amyrin derivative was further shown by its behavior on oxidation with permanganate in acetic acid solution. Here again two neutral sulfur-free oxidation products were formed, which were shown to be respectively $C_{31}H_{46}O_4$ and $C_{31}H_{44}O_5$ and were apparently analogous to the substances obtained from the β-amyrin thio compound. The former proved to be a ketone since it yielded a monooxime. Attempts to acylate this ketone were not successful, leaving for the moment the nature of the other oxygen atom undetermined. The separation of this substance from the other oxidation products afforded considerable difficulty which probably accounts for the deviation from the theory of the analytical data obtained from it and its derivatives. The second oxidation product, also of neutral character, did not yield an oxime and could not be acylated but was shown by its behavior towards alkali to be in all likelihood a lactone. But in the study of the action of alkali on these substances it was found necessary to take into consideration a complicating factor in the form of the methyl ester group.

In previous papers on hederagenin methyl ester and its derivatives, the stability and resistance to saponification of this ester group have been reported.[3] In view of the observations given below, we have studied again the behavior towards alkali of hederagenin methyl ester itself and certain of its derivatives. Although as originally reported, the ester group of these substances is unusually resistant to the saponifying action of alkali, conditions have now been found for their saponification. This was accomplished by heating the esters for 3 to 5 hours in 10 per cent alcoholic potassium hydroxide at 130°. It was possible then to isolate in each case about 50 per cent of the free acids along with unchanged ester.

On turning to the study of the action of alkali on the thio compound described above, the resistant property of the methyl ester group was found to be retained. In contrast to this, however, the methyl ester groups of the two oxidation products derived from the sulfur compound were found to be surprizingly easily saponified.

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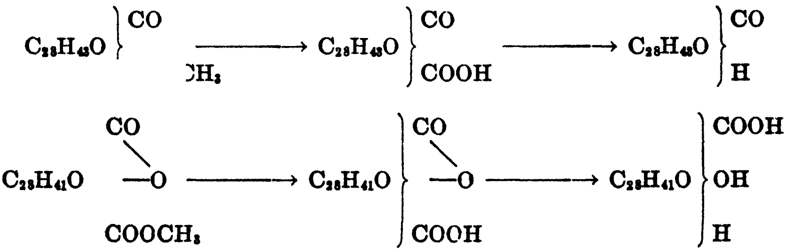
[3] Jacobs, W. A., *J. Biol. Chem.*, **63**, 628 (1925). van der Haar, A. W., *Rec. trav. chim. Pays-Bas*, **44**, 740 (1925).

This was quantitatively accomplished by refluxing these substances in dilute alcoholic alkali which was the equivalent of 0.05 N From each substance, $C_{31}H_{46}O_4$ and $C_{31}H_{44}O_5$, were obtained the free acids, $C_{30}H_{44}O_4$ and $C_{30}H_{42}O_5$, respectively. Since the sulfur compound itself cannot be similarly saponified, it is apparent that the sulfur atom must be situated in the same general vicinity in the molecule as the carbomethoxyl group in order that its removal on oxidation with the introduction of oxygen atoms should labilize this ester group. This conclusion was further supported by the observation that the carboxyl group itself which was originally present in hederagenin and is now liberated again by saponification of the ester group displays a new kind of lability not previously noted in hederagenin and its derivatives. On heating the above keto ester, $C_{31}H_{46}O_4$, with 10 per cent potassium hydroxide at 130°, a neutral substance was obtained which was found to possess the formula $C_{29}H_{44}O_2$. This substance owed its origin to the loss of $CO_2$ from the carboxyl group following its preliminary liberation by saponification from the ester group.

The second oxidation product, $C_{31}H_{44}O_5$, which has been described as a lactone, exhibited a similar behavior but in this case the lactone group was simultaneously saponified. Therefore, the opening of the lactone group together with saponification of the methyl ester group and simultaneous loss of $CO_2$ from the carboxyl group liberated in the latter case resulted in an acid substance, $C_{29}H_{44}O_4$. In the case of the keto ester and the lactone ester these reactions may be represented as follows:



$$C_{28}H_{43}O\left\}\begin{array}{l}CO\\ \\ CH_3\end{array}\right. \longrightarrow C_{28}H_{43}O\left\}\begin{array}{l}CO\\ \\ COOH\end{array}\right. \longrightarrow C_{28}H_{43}O\left\}\begin{array}{l}CO\\ \\ H\end{array}\right.$$

$$C_{28}H_{41}O\ \begin{array}{l}CO\\ \quad\diagdown\\ —O\\ \\ COOCH_3\end{array} \longrightarrow C_{28}H_{41}O\left\}\begin{array}{l}CO\\ \quad\diagdown\\ —O\\ \\ COOH\end{array}\right. \longrightarrow C_{28}H_{41}O\left\}\begin{array}{l}COOH\\ \\ OH\\ \\ H\end{array}\right.$$

The formation of these substances, therefore, involves the loss of the original carboxyl group of hederagenin.

The lability of the ester carboxyl in these oxidation products suggests that the ketone $C_{31}H_{46}O_4$, is a $\beta$-keto ester and that in the ester lactone, $C_{31}H_{44}O_5$, the lactone group is also in a $\beta$ position to the methyl ester group. From these facts it may be suggested with reservation that in the thio compound one of the carbon atoms to which the sulfur atom is attached is $\beta$ to the methyl ester group. On removal of the sulfur atom by oxidation, this carbon atom now becomes carbonyl in the case of keto ester and a lactone carbon in the case of the second oxidation product. Although it is possible to suggest explanations of the formation of such oxidation products from an assumed polycyclic thiophene derivative, we do not feel that this is justified until more data have been obtained.

The formation of the sulfur compounds and their behavior on oxidation in both $\beta$-amyrin and hedraganic methyl ester present exactly analogous pictures which very strongly suggest that identical structures are involved in each case. A study of this reaction is being extended to include other sapogenins, triterpene alcohols, etc.

## EXPERIMENTAL.

*Dehydrogenation of Hedraganic Methyl Ester.*—A mixture of 5 gm. of hedraganic methyl ester[2] and an equal amount of sulfur was heated in an atmosphere of nitrogen for 5 hours, at a temperature of 210–220°. The reaction mass was extracted with ether and the filtered solution was evaporated to dryness. The resulting solid was distilled at 3 mm. from a 10 cc. Claisen distilling flask. The temperature of the metal bath, in which the flask was immersed, was gradually increased until a temperature of 275° was attained. At this point practically all of the free sulfur had distilled. A cherry-colored resin distilled when the temperature of the bath was maintained between 300–350°. A solution of this resin in 10 cc of hot acetone gradually crystallized The collected substance was then recrystallized from acetone and finally from alcohol It separated in the form of colorless prisms which melted at 137–138°. In pyridine solution no appreciable optical activity could be noted.

For analysis the substance was recrystallized four times from acetone. Following this, in order to confirm the analytical data the substance was again recrystallized four times from acetone.

(*a*) 5.880 mg. substance*: 4.700 mg. $H_2O$, 16.665 mg. $CO_2$.
(*b*) 4.668 " " *: 3.837 " " 13.260 " "
(*c*) 4.508 " " †: 3.740 " " 12.810 " "
(*d*) 5.220 " " †: 4.210 " " 14.810 " "
(*e*) 7.425 " " : 3.460 mg. $BaSO_4$.

| | | | C | H | S |
|---|---|---|---|---|---|
| $C_{31}H_{44}O_2S$. | Calculated. | | C 77.45, | H 9.16, | S 6.67. |
| $C_{30}H_{42}O_2S$. | | | 77.20, | 9.08, | " 6.8 |
| | Found. | (*a*) | 77.30, | 8.95. | |
| | | (*b*) | 77.46, | 9.19. | |
| | | (*c*) | 77.50, | 9.28. | |
| | | (*d*) | 77.38, | 9.03. | |
| | | (*e*) | | | S 6.43. |

* Recrystallized four times.
† Recrystallized eight times.

Titration showed no free acidity. In an experiment in which the substance was refluxed for 5 hours with 1 per cent alcoholic KOH, 50 per cent of recrystallized starting material was recovered and only an inappreciable amount of acid product was obtained. The methyl ester group, as in the starting material, is thus seen to be difficult to saponify. The cholesterol test gave an orange solution which changed to a deep red when warmed. A molecular weight determination according to the method of Rast gave the following result:

14.587 mg. camphor: 2.242 mg. substance, $\Delta = 14.5°$. Mol. wt., calculated, 476.4; found, 450.

### *Oxidation of the Thio Compound.*

*The Lactone Ester, $C_{31}H_{44}O_5$.*—A solution of 3 gm. of the above sulfur compound in 300 cc. of acetic acid was stirred while 125 cc. of 6 per cent $KMnO_4$ solution were added. After stirring for 1 hour, the solution was diluted with 3 volumes of water. The white precipitate which separated was removed from the colloidal solution of $MnO_2$ by filtration. Final traces of $MnO_2$ were eliminated by dissolving the precipitate in alcohol and filtering the solution. The alcoholic filtrate was evaporated to dryness and the residue was dissolved in ligroin (80–90°). This solution was then concentrated on a steam bath to a volume of 300 cc. The cooled solution deposited long, needle-shaped crystals. When this product was recrystallized from acetone it melted at 274–275°. The cholesterol test gave a yellow color which deepened to an amber when the solution was warmed.

−16° (c = 1.017 in pyridine).

5.130 mg. substance 4.022 mg. $H_2O$, 14.070 mg. $CO_2$.
4.262 " " : 3.400 " " 11.642
5.490 " " : 2.692 " AgI.
$C_{31}H_{44}O_5$. Calculated. C 74.96, H 8.92, $OCH_3$ 6.25.
$C_{30}H_{42}O_5$. " " 74.70, " 8.72, " 6.44.
Found. (a) " 74.80, " 8.77.
(b) " 74.52, " 8.92.
(c) $OCH_3$ 6.47.

Contrary to the original thio compound and hederagenin methyl ester, the ester group of this oxidation product is easily saponified by dilute alkali as follows:

*Saponification of the Methyl Ester Group of the Lactone Ester, $C_{31}H_{44}O_5$.*—15.372 mg. of substance were dissolved in 4 cc. of alcohol. The solution showed no free acidity. 3.203 cc. of 0.1 N NaOH were added and the mixture was refluxed in an atmosphere of nitrogen for 4 hours, and then titrated back. Found, 0.239 cc. Calculated for 1 equivalent, 0.262 cc.

The solution on acidification with acetic acid and concentration yielded the crystalline lactone acid. When this product was recrystallized from diluted acetone, it separated in the form of plates which melted at 255–256° with decomposition.

4.370 mg. substance: 3.380 mg. $H_2O$, 11.900 mg. $CO_2$.
$C_{30}H_{42}O_5$. Calculated. C 74.62, H 8.79.
$C_{29}H_{40}O_5$. " " 74.31, " 8.60.
Found. " 74.27, " 8.65.

*Cleavage of the Lactone Ester, $C_{31}H_{44}O_5$, with Strong Alkali.*—A solution of 0 2 gm of the lactone ester in 15 cc. of 10 per cent alcoholic KOH was heated in a sealed tube at 130–140° for 4 hours The solution was diluted with 3 volumes of water and the small amount of precipitate which separated was filtered off. The filtrate was acidified and the acid product which precipitated was collected with water. When recrystallized from acetone it separated in the form of hexagonal plates which melted at 209–210°.

4.900 mg. substance: 4.265 mg. $H_2O$, 13.585 mg. $CO_2$.
3.660 " " : 3.070 " " 10.180 " "
$C_{29}H_{44}O_4$. Calculated. C 76.25, H 9.73.
$C_{28}H_{42}O_4$. " " 75.95, " 9.58.
Found. (a) " 75.61, " 9.74.
(b) " 75.86, " 9.39.

10.355 mg. substance were dissolved in 4 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found, 0.250 cc. Calculated for 1 equivalent, 0.227 cc.

In attempts to prepare a benzoate and an oxime of the lactone ester it was recovered unchanged.

*The Keto Ester*, $C_{31}H_{46}O_4$.—This product of the oxidation of the thio compound was obtained from the ligroin mother liquor of the first crystallization of the above lactone ester. When this solution was concentrated to about 25 cc. long, fibrous needles separated on cooling. Purification of this substance was accomplished by repeated fractionation from ether, in which the lactone ester is much less soluble This separation proved to be very difficult and it is possible that the substance was still not homogeneous. This may account in part for the rather unsatisfactory analytical figures obtained with this and its succeeding derivatives. Final purification was attempted by dissolving the substance in ether and inducing crystallization by the addition of petrolic ether. The silky needles obtained melted at 202–204°.

$[\alpha]_D^{28}$ = −184° (c = 1.027 in pyridine).
5.420 mg. substance: 4.385 mg. $H_2O$, 15.240 mg. $CO_2$.
4.280 " " : 3.470 " " 12.055 " "
5.160 " " : 2.505 " AgI.
$C_{31}H_{46}O_4$. Calculated. C 77.11, H 9.62, $OCH_3$ 6.44.
$C_{30}H_{44}O_4$. " " 76.85, " 9.41, " 6.63.
Found. (*a*) " 76.69, " 9.05.
(*b*) " 76.83, " 9.06.
(*c*) $OCH_3$ 6.41.

In an attempt to benzoylate the keto ester it was recovered unchanged.

*The Oxime of the Keto Ester*, $C_{31}H_{46}O_4$.—A mixture of 0.1 gm. of the keto ester and hydroxylamine in absolute alcoholic solution was heated in a sealed tube at a temperature of 110–120° for 10 hours. The alcoholic solution was then concentrated and the oxime was allowed to crystallize. Repeated recrystallizations from alcohol gave a substance which melted at 236–237° with decomposition.

4.730 mg. substance: 3.940 mg. $H_2O$, 13.045 mg. $CO_2$.
4.000 " " : 3.200 " " 11.045 " "
7.450 " " : 0.196 cc. N (26°, 761.5 mm.).
4.903 " " : 2.560 mg. AgI.
$C_{31}H_{47}O_4N$. Calculated. C 74.79, H 9.54, N 2.82, $OCH_3$ 6.23.
$C_{30}H_{45}O_4N$. " " 74.47, " 9.39, " 2.89, " 6.41.
Found. (*a*) " 75.22, " 9.32.
(*b*) " 75.28, " 8.95.
(*c*) N 3.00.
(*d*) $OCH_3$ 6.89.

*Saponification of the Methyl Ester Group of the Keto Ester, $C_{31}H_{46}O_4$.*—10.867 mg. of substance were dissolved in 4 cc. of alcohol. The solution showed no free acidity. 3.222 cc. of 0.1 N NaOH were added and the solution was refluxed in an atmosphere of nitrogen for 4 hours and then titrated back. Found, 0.252 cc. Calculated for 1 equivalent, 0.242 cc.

When the solution was acidified with acetic acid and then concentrated, crystallization occurred. When this product was recrystallized from dilute acetone it separated in the form of fine needles which melted at 184–185°with decomposition.

3.987 mg. substance: 3.270 mg. $H_2O$, 11.222 mg. $CO_2$.
3.955 " " : 3.110 " " 11.167 " "
$C_{30}H_{44}O_4$. Calculated. C 76.85, H 9.41.
$C_{29}H_{42}O_4$. " " 76.60, " 9.32.
Found. (*a*) " 76.76, " 9.18.
(*b*) " 77.00, " 8.80.

*Cleavage of the Keto Ester, $C_{31}H_{44}O_4$, with Strong Alkali.*—A solution of 0.2 gm. of the ketone in 15 cc. of 10 per cent alcoholic potassium hydroxide solution was heated in a sealed tube at 130–140° for 3½ hours. The solution was diluted with 3 volumes of water and the precipitate was collected with water. The dried material was extracted with a small amount of petroleum ether in order to remove a small amount of non-crystalline by-product. When the residue was recrystallized from ether it separated in the form of needles which melted at 262–264° and proved to be neutral.

4.100 mg. substance: 3.660 mg. $H_2O$, 12.415 mg. $CO_2$.
3.595 " " : 3.170 " " 10.900 " "
$C_{29}H_{42}O_2$. Calculated. C 82.41, H 10.03.
$C_{28}H_{40}O_2$. " " 82.35, " 9.88.
Found. (*a*) " 82.61, " 9.99.
(*b*) " 82.69, " 9.87.

*Saponification of Hederagenin Methyl Ester.*—A solution of 0.2 gm. of the ester in 10 cc. of 10 per cent alcoholic KOH was heated in a sealed tube at 120–130° for 5 hours. Dilution with 3 volumes of water gave a precipitate which was collected and dried. The dried material was extracted with ether, leaving the insoluble potassium salt of hederagenin. The latter was obtained by liberation with acid and recrystallization from alcohol. The resulting substance agreed in all properties with hederagenin.

4.050 mg. substance: 3.800 mg. $H_2O$, 11.358 mg. $CO_2$.
$C_{31}H_{50}O_4$. Calculated. C 76.48, H 10.36.
Found. " 76.49, " 10.50.

The ether extract of the above potassium salt yielded 60 mg. of residue which after recrystallization was shown to be unchanged starting material.

*Saponification of Hedraganic Methyl Ester.*—A solution of 0.1 gm. of the methyl ester in 10 cc. of 10 per cent alcoholic KOH was heated at 120–130° for 3 hours. The solution was then diluted with 3 volumes of water and the precipitate was collected with water. The dried precipitate was extracted with dry ether. The insoluble residue of potassium salt was suspended in ether and shaken with dilute HCl. When complete solution had occurred due to the liberation of the free acid, the ether solution was washed repeatedly with water and then concentrated. The residue was recrystallized from acetone by the addition of water. Hedraganic acid formed needles which melted at 242–243°.

2.540 mg. substance: 2.440 mg. $H_2O$, 7.637 mg. $CO_2$.
$C_{30}H_{48}O_2$. Calculated. C 81.75, H 10.99.
Found. " 82.00, " 10.72.

The above ether extract of the potassium salt on evaporation gave a neutral residue which was recrystallized from acetone. This fraction was identified by melting point and mixed melting point as starting material.

# A NOTE ON THE CALCIUM RETENTION ON A HIGH AND LOW FAT DIET.

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Most of the reports of investigations on the subject of the relation of fat to calcium retention deal with animal experiments (1–3) or are studies on infants or small children (4–9). The results reported by these investigators are often conflicting. The literature reported upon the subject of the relation of fat *per se* to calcium retention in adult human subjects is limited, although a few investigators (10, 11) suggest that there may be some relationship between fat and calcium retention. Stewart and Percival (12) have summed up the situation in the following statement: "It has often been stated that the absorption of calcium is greatly aided by the addition of fat to the diet, and equally often it has been denied that fat has any such effect."

The object of this study was to secure data upon the calcium retention of women on a diet relatively high in fat and also on a diet low in fat.

## EXPERIMENTAL.

The diet given in Table I was planned to contain either a relatively high or a very low amount of fat, to furnish from 0.45 to 0.50 gm. of calcium daily which is an amount close to the estimated minimum requirement at which calcium equilibrium might be expected (13), to eliminate as far as possible any vitamin problem, and to meet the protein and energy requirements of the two healthy young women who were the subjects of the experiment.

Raw rather than pasteurized milk (14, 15), centrifuged under the supervision of the Department of Dairy Husbandry, furnished 94 per cent of the calcium of the diet. The daily allowance of milk, 300 gm., was determined by preliminary calcium analyses of the milk of the Jersey cow which furnished the milk for the experiment.

The average daily intake of calcium for each subject was 9.6 mg. per kilo of body weight. This amount was sufficient for calcium equilibrium for women subjects as indicated by the work of other investigators (16, 17). The average daily fat intake on the high fat diet was 105.6 gm. per subject and on the low fat diet only 5.8 gm.

TABLE I.

*Daily Food Intake.*

| | High fat diet. | | | | | Low fat diet. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Weight of food. | Calcium. | | Fat. | | Weight of food. | Calcium. | | Fat. | |
| | | Period I. | Period II. | Period I. | Period II. | | Period I. | Period II. | Period I. | Period II. |
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| Milk, centrifuged | 300 | 0.462 | 0.441 | 0.30 | 0.27 | 300 | 0.459 | 0.459 | 0.09 | 0.18 |
| Lard | 100 | | | 100.00 | 100.00 | | | | | |
| Corn-starch | 50 | 0.001 | 0.001 | | | 60 | 0.001 | 0.001 | | |
| Sugar, cane | 115 | | | | | 330 | | | | |
| Apples, cored but unpared | 400 | 0.019 | 0.019 | 2.00* | 2.00* | 400 | 0.018 | 0.018 | 2.00* | 2.00* |
| Beef, lean, free from visible fat | 150 | 0.013 | 0.013 | 3.32 | 3.32 | 150 | 0.007 | 0.007 | 3.74 | 3.74 |
| Sodium chloride, C.P.† | 5 | | | | | 5 | | | | |
| Total | | 0.495 | 0.474 | 105.62 | 105.59 | | 0.485 | 0.485 | 5.83 | 5.92 |

* Rose, M. S., Laboratory handbook for dietetics, 3rd edition, New York (1929).

† Calcium content of the NaCl, C.P., was determined and found to be 0.00002 gm. per 5 gm.

## *Procedure.*

The diet was eaten for a period of 18 consecutive days during the late winter. The 18 days were divided into two periods of 9 days each, one for the high fat and the other for the low fat diet. Each 9 day period consisted of a 3 day preliminary period followed by two 3 day experimental periods. During the preliminary period the same weighed amounts and kinds of food were eaten as those of the experimental diet. The calcium intake for both the high fat and the low fat diet was practically the same, averaging 0.484 and 0.485 gm. respectively. The subjects worked indoors during this study and were not exposed to direct sunlight. They performed all chemical analyses in the Nutrition Laboratory and also prepared the food of the diet.

A composite sample of the centrifuged milk for every 3 days was used for analysis for calcium and fat. The food and feces were ashed (18) in an electric muffle furnace at 400–450°. Calcium was determined in all of the foods except cane-sugar and lard.

Urine and feces of the 3 day preliminary period were not collected. The first collection of the excreta was made on the 1st day of the experimental period.

### *Method of Analysis.*

Calcium of food, feces, and urine was determined by McCrudden's method (19) with the pH value adjusted according to Shohl and Pedley (20). The recommendations of Halverson and Bergeim (21) were followed in regard to the amount of ammonium oxalate used and in the preparation and standardization of the 0.01 N and 0.05 N $KMnO_4$ solutions. Determinations of calcium and fat were made at least in duplicate and most of them in triplicate.

The fat content of the centrifuged milk was determined by the Babcock method (22) and the fat of the beef by means of the Soxhlet fat extraction apparatus (23).

## DISCUSSION AND RESULTS.

The subjects in this study were nutrition students who had lived on an adequate diet previous to this study. They were well within the correct range of weight for their height and lived healthy active lives.

The average daily calcium intake and output for the subjects for both the high and low fat diets are given in Table II.

On the *high fat* diet the calcium balance for both subjects shifted from positive to negative, or *vice versa.* Subject R. J. showed a positive calcium balance at the end of the first 3 day period and a negative one at the end of the following 3 day period. Subject M. J. on the same diet showed the same result but the order was reversed, as indicated in Table II.

On the *low fat* diet Subject R. J. showed a negative calcium balance for the two 3 day periods, with a tendency toward equilibrium during the second 3 day period. Subject M. J. changed from a very slightly negative balance to a positive balance.

TABLE II.

*Calcium Balance (Average Daily) for High and Low Fat Diets.*

| Subject. | Period No. | Calcium intake. |  | Calcium output. |  |  | Balance. | Balance per kilo body weight. |
|---|---|---|---|---|---|---|---|---|
|  |  | Total. | Per kilo body weight. | Urine. | Feces. | Total. |  |  |
| High fat diet. |  |  |  |  |  |  |  |  |
|  |  | *gm.* | *mg.* | *gm.* | *gm.* | *gm.* | *gm.* | *mg.* |
| R. J., weight | I | 0.495 | 9.8 | 0.113 | 0.335 | 0.448 | +0.047 | +0.9 |
| 50.6 kilos. | II | 0.474 | 9.4 | 0.111 | 0.463 | 0.574 | −0.100 | −1.9 |
| M. J., weight | I | 0.495 | 9.8 | 0.116 | 0.421 | 0.537 | −0.042 | −0.8 |
| 50.3 kilos. | II | 0 474 | 9 4 | 0.128 | 0.295 | 0.423 | +0.051 | +1.0 |
| Average |  | 0 484 | 9.6 |  |  |  |  |  |
| Low fat diet. |  |  |  |  |  |  |  |  |
| R. J. | I | 0.485 | 9 6 | 0.105 | 0.532 | 0.637 | −0.152 | −3.0 |
|  | II | 0.485 | 9.6 | 0.125 | 0.419 | 0.544 | −0.059 | −1.2 |
| M. J. | I | 0.485 | 9.6 | 0.154 | 0.339 | 0.493 | −0.008 | −0.2 |
|  | II | 0.485 | 9.6 | 0.157 | 0.281 | 0.438 | +0.047 | +0.9 |
| Average |  | 0.485 | 9.6 |  |  |  |  |  |

As the subjects shifted from a positive to a negative calcium balance or *vice versa* within two 3 day periods it would seem that fat *per se* cannot be said to have exercised a definite influence upon the calcium retention of the two subjects of this study.

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# STUDIES ON CEREBROSPINAL FLUID AND SERUM CALCIUM, WITH SPECIAL REFERENCE TO THE PARATHYROID HORMONE.*

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The problem as to the condition in which calcium exists in the blood plasma has received considerable attention but it is exceedingly difficult to arrive at a definite conclusion from the published accounts. Efforts to determine the calcium ion concentration by the potentiometric method have so far not been successful, but it is estimated that about 2.5 mg. per cent of calcium is in the ionized state. The situation is not much more encouraging when we consider the diffusible and non-diffusible fractions of the serum or plasma calcium, because the results of different investigators are so much at variance and the methods of fractionating the calcium, namely compensation dialysis and ultrafiltration, are not above criticism from a theoretical point of view.

Cameron and Moorhouse (4) in a recent study proceed on the assumption that the cerebrospinal fluid calcium represents the true diffusible calcium of the blood plasma. Fremont-Smith(10) points out that neither the dialysis nor the secretion hypothesis of cerebrospinal fluid formation can be accepted unqualifiedly, though the evidence as a whole seems to be in favor of the assumption that the process is one of dialysis. Walter (24) expresses a similar view in his recent monograph. It is questionable, however, if the facts fully justify the idea of a "simple membrane equilibrium existing between plasma and cerebrospinal fluid," as is maintained by Mestrezat (14), Pincus and Kramer (19), etc.

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* The experimental results of this paper are taken from the thesis submitted by the junior author in partial fulfilment of the requirements for the Degree of Master of Arts in Biochemistry.

Updegraff, Greenberg, and Clark (23) also point out certain difficulties in applying the Donnan membrane equilibrium to the plasma-cerebrospinal fluid system.

Osnato, Killian, Garcia, and Mattice (18) in summarizing the work done by means of ultrafiltration and of compensation dialysis give values for the diffusible fraction of the plasma calcium which cover a range of 39 to 75 per cent of the total. These values are not only very variable but also considerably higher than those obtained from the cerebrospinal fluid. The results obtained from the study of cerebrospinal fluid calcium, on the contrary, are marked for their small variability. In a number of dispensary patients we found that the ratio of cerebrospinal fluid calcium (4.8 to 5.0 mg. per cent) to total serum calcium varied from 46 to 51 per cent, with an average of 48.6 per cent.[1] Barrio (2) in syphilitic patients found that the cerebrospinal fluid calcium was 45 to 57 per cent of the serum calcium; Cantarow (5) in a large number of normal individuals also found the ratio to vary between 45 and 55 per cent. In twenty-two normal dogs we determined simultaneously the calcium of the serum and cerebrospinal fluid, the latter ranging within the narrow limits of 5.2 to 5.9 mg. per cent. In 80 per cent of our analyses the ratio between cerebrospinal fluid and serum calcium was 46.3 to 53.2 per cent (average 50.0 per cent). Our results are somewhat lower than those reported by Cameron and Moorhouse (4) who found 4.8 to 6.3 mg. per cent of calcium in the cerebrospinal fluid of normal dogs with an average ratio of 53 per cent of the total plasma calcium.

### *Experimental Changes in Cerebrospinal Fluid Calcium.*

Assuming that the cerebrospinal fluid calcium represents a dialyzate from the blood plasma, we attempted to study the variation in its calcium level with changing concentrations in the blood calcium.

### *A. Ingestion of Calcium Salts.*

Several attempts have been made to increase the serum calcium content of normal subjects by means of oral administration of

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[1] All calcium determinations were made according to the procedure of Clark and Collip (6).

calcium salts, but the results are rather inconstant. Some investigators report that they have not been able by this method to produce any increase in the serum calcium of either man or certain of the laboratory animals; some have produced a slight temporary rise, while others have reported a very marked temporary increase. Denis and Minot (8) report that in most cases it is impossible to increase the serum calcium by the ingestion of calcium salts. Howland and Kramer (12) working with rats and Clark with rabbits obtained similar results. In these experiments, however, the blood was not studied at very short intervals following the calcium administration, and the calcium salts were in some cases given with the regular diet. Bauer and Ropes (3) administered calcium lactate in 5 to 10 gm. doses to normal subjects 12 hours after the last meal, and observed a maximum rise of 28 per cent between the 1st and 5th hour, and in some instances this elevation was maintained for 12 hours. Kahn and Roe (13), on the other hand, observed very large increases in the normal fasting subjects. In one of their studies they report that a 20 gm. dose caused an 81 per cent increase in the serum calcium within 4 to 5 hours; 5 gm. gave a rise of 80 per cent in 6 or 7 hours and 2 gm., 41 per cent increase within 6 hours. In another investigation they observed a rise of 108 per cent after 5 gm. of calcium lactate were administered.

We fed Dog 24 excessive amounts of calcium lactate and calcium chloride in his food for several days, but the serum calcium did not show any increase. In the case of Dog 27, 5 gm. of calcium lactate were administered by stomach tube following a 24 hour fast, and within 3 hours the serum calcium had increased 12 per cent and was still maintained at this level after 6 hours. Dog 26 also received 5 gm. of calcium lactate and showed an increase in the serum calcium of 18 per cent after 2 hours and 11 per cent after 6 hours. It is important, however, to note that although the findings are variable as to the magnitude of the rise, the increase in the serum calcium when produced by the ingestion of calcium salts is only of short duration and usually there is a return to the normal level within 12 to 15 hours.

In no instance did we observe any appreciable alteration in the cerebrospinal fluid calcium corresponding to the change in serum calcium. This is more obvious in the experiments where the

TABLE I.

*Administration of Parathyroid Hormone and Calcium Salts.*

| Dog No. | Date. | Plasma volume. | Ca per 100 cc. | | | | | $\frac{\text{C.S.F.Ca*}}{\text{Serum Ca}} \times 100$. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | | Serum. | Plasma. | Blood. | Blood (calculated). | Cerebrospinal fluid. | | |
| | *1928* | *per cent* | *mg.* | *mg.* | *mg* | *mg.* | *mg* | | |
| 10 | Mar. 27 | 59.9 | 10.8 | 10.4 | 6.5 | 6.5 | 5.5 | 50.9 | Normal. |
| | " 29 | 58.0 | 14.1 | 13 7 | 8.9 | 8.2 | | | Preceding day 5 cc. paroidin injected in 4 doses at 3 hr. intervals. |
| | Apr. 5 | 64.8 | 12.5 | 11.9 | 8.1 | 8.1 | 5.9 | 47.1 | Preceding evening 5 cc. paroidin injected in 1 dose. |
| | May 3 | 47.8 | 11.0 | 10.5 | | | 5.5 | 50.4 | Normal. |
| | " 7 | 52.0 | 17.3 | 15.9 | 9.7 | 8.7 | 6.1 | 35.2 | Food for previous 4 days contained much $CaCl_2$ and lactate. Preceding day 2 injections of 5 cc. each of paroidin about 5 hrs. apart. |
| 15 | " 3 | 42.5 | 10.5 | 9.7 | | | 5.2 | 49.4 | Normal. |
| | " 7 | 48.7 | 15.5 | 14.6 | 7.7 | 7.6 | 6.3 | 40.5 | Food for previous 4 days contained much $CaCl_2$ and lactate. Preceding day 2 injections of 5 cc. each of paroidin about 5 hrs. apart. |
| 24 | " 11 | 54.3 | 10.6 | 9.8 | | | 5.7 | 54.2 | Normal. |
| | " 17 | 49.1 | 10.6 | 9.9 | | | 5.5 | 52.0 | About 60 gm. of equal parts $CaCl_2$ and lactate given with food during preceding 6 days. |
| | " 21 | 43.5 | 16.0 | 15.0 | | | 6.3 | 39.6 | During 2 days preceding 13 cc. paroidin administered in 3 injections, and 10 gm. $CaCl_2$ by stomach tube. |
| | " 22 | | 14.0 | | | | 6.0 | 42.9 | |
| | " 23 | | 11.0 | | | | 5.7 | 51.6 | |
| 23 | " 2 | 51.3 | 11.7 | 10.6 | | | 5.7 | 49.0 | Normal. |
| | " 5 | 49.8 | 16.6 | 16.4 | 9.6 | 8.3 | 6.5 | 39.4 | Previous 3 days total of 25 gm. $CaCl_2$ and lactate given in food. Day previous 7 cc. parathormone in 3 injections. |

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* C.S.F.Ca indicates cerebrospinal fluid calcium.

TABLE I—*Concluded.*

| Dog No. | Date. | Plasma volume. | Ca per 100 cc. | | | | | $\frac{\text{C.S.F.Ca*}}{\text{Serum Ca}} \times 100$. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | | Serum. | Plasma. | Blood. | Blood (calculated). | Cerebrospinal fluid. | | |
| | *1928* | *per cent* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | | |
| 26 | May 24 | | 11.8 | | | | 5.9 | | Normal. |
| | | | 14.1 | | | | | | 5 gm. Ca lactate by stomach tube; blood samples for analysis at 2 hr. intervals. |
| | | | 13.0 | | | | | | |
| | | | 13.1 | | | | 5.8 | 44.2 | |
| 27 | " 27 | | 10.5 | | | | 5.3 | | |
| | | | 12.0 | | | | | | 5 gm. Ca lactate by stomach tube; blood sample at 3 hr. intervals. |
| | | | 12.0 | | | | 5.6 | 46.6 | |

feeding of calcium salts was reinforced with subcutaneous injections of parathyroid hormone. In this case, of course, we secured considerable rises in the serum calcium (16 to 17 mg. per cent) but the cerebrospinal fluid calcium under these conditions increased only slightly above its normal range so that the ratio of cerebrospinal fluid calcium to serum calcium fell considerably below the normal range of its variation. Dog 24 presents an interesting condition. Under forced feeding of calcium salts there was no alteration either in the cerebrospinal fluid or serum calcium, but following injections of paroidin while the calcium salts were administered the serum calcium was built up to a level of 16 mg. per cent. The cerebrospinal fluid Ca in the meantime increased to 6.3 mg. per cent so that the ratio diminished to only about 40 per cent. We followed the changes in serum and cerebrospinal fluid calcium in this dog for 3 days, and found that the serum calcium was diminishing relatively more rapidly than the cerebrospinal fluid calcium so that the ratio gradually increased, reaching the normal value at the end of the 3rd day (51.6 per cent). (See Table I.)

### *B. Effect of Bile.*

Emerson (9) studied the calcium of blood and tissues in jaundiced dogs and dogs with bile fistula, and reported a marked decrease in the serum calcium, the whole blood calcium, and a very marked decrease in the diffusible calcium in dogs jaundiced over a period of 40 days. In one animal the decrease in serum calcium amounted to 22 per cent at the end of 31 days and in another a 28 per cent decrease was observed. According to his findings, by the use of the method of ultrafiltration, the diffusible calcium in one case fell as low as 0.8 mg. per cent. In the dogs with bile fistula the changes were quite the reverse. Over a period of 79 days there was a marked increase in the calcium of the serum and whole blood and a very marked rise in the diffusible calcium up to 8.6 mg. per cent.

In our studies we ligated and sectioned the common bile ducts of two dogs and determined the blood serum calcium at intervals following the operation. In our experiments, though a definite jaundiced condition developed, the serum calcium concentration did not undergo any striking reduction. In Dog 32 there was a 10 per cent decrease after 33 days and in Dog 33 the normal level was still maintained 33 days after the operation. The cerebrospinal fluid calcium remained unchanged. Our results are fully corroborated by the recent findings of Snell and Greene (22) who likewise find no disturbance in serum calcium in conditions of clinical or experimental (dog) jaundice.

### *C. Parathyroidectomy.*

Following complete extirpation of the parathyroid glands we invariably observed a marked decrease in the serum calcium, the concentration falling in one instance below 5 mg. per cent. These findings are in accord with the observations of numerous other investigators. There does not seem to be any definite level of serum calcium at which tetany develops, although in the literature references may be seen to the "tetany level." In our experience the symptoms may appear when the calcium is over 8 mg. per cent, and Cameron and Moorhouse (4) in their paper report a case of severe tetany with a serum calcium of 8.7 mg. per cent. On the other hand, one of our animals, Dog 5, had a severe attack 13 days after operation, when the calcium was 5.3 mg. per cent. The animal was kept symptom-free for several days by injections of parathyroid extract. The treatment was then discontinued and it lived for 24 days showing only very slight and transient symptoms although 3 weeks after the last injection of the extract the serum calcium was only 3.5 mg. per cent.

In a number of thyroparathyroidectomized dogs, we studied simultaneously the calcium of serum, citrated plasma, and whole blood and noted also the relative cell volume. The differences between the serum calcium and the plasma calcium in nine parathyroidectomized dogs were very slight and in a total of thirteen analyses they ranged from 0 to 0.8 mg. per cent. We observed about the same deviation in the normal dog and, therefore, may conclude from our findings that following parathyroidectomy, the plasma and serum undergo the same change in calcium content. The plasma calcium is invariably somewhat lower than the serum calcium and this is due to the use of the anticoagulant which causes shrinkage of the cells with resulting dilution of the plasma. It is possible by varying the concentration of the anticoagulant (lithium citrate) to obtain variable results for the plasma volume per cent as well as for the plasma calcium concentration, the former increasing and the latter diminishing. The decrease in calcium is, however, directly proportional to the concentration of the anticoagulant. From experimental studies on the effect of this factor we know that our plasma values should be about 5 to 6 per cent lower than the serum values, and if such a correction is made the two become practically the same. These observations, however, are quite contradictory to the findings of Cameron and Moorhouse (4), who state that in acute parathyroid tetany they found the blood plasma to contain 3 or 4 mg. per cent *more* calcium than did the serum, which we cannot help but feel is entirely erroneous.

The study of cerebrospinal fluid calcium in the parathyroidectomized dogs reveals some interesting facts. In all our determinations we find that the cerebrospinal fluid calcium does not remain stationary but as the serum calcium diminishes it also invariably falls though not to the same extent, so that the ratio of cerebrospinal fluid calcium to the serum calcium increases far beyond its normal range. In no instance, however, did we observe the condition where the two became actually the same. In other words, we never obtained a ratio of practically 100 per cent, as Cameron and Moorhouse report, the highest ratio in our experience being 93.5 per cent (Table II). No matter how low the

TABLE II.

*Parathyroidectomy.*

| Dog No. | Date. | Plasma volume. | Ca per 100 cc. | | | | | $\frac{\text{C.S.F.Ca*}}{\text{Serum Ca}} \times 100.$ | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | | Serum. | Plasma. | Blood. | Blood (calculated). | Cerebrospinal fluid. | | |
| | *1928* | *per cent* | *mg.* | *mg.* | *mg* | *mg.* | *mg* | | |
| 17 | Apr. 6 | 48.9 | 12.0 | 10.9 | 6.0 | 5 8 | | | Normal. |
| | " 9 | 50.4 | 5 4 | 5.1 | 3.4 | 2.7 | | | Severe tetany, 3 days after operation. |
| 18 | " 9 | 57.4 | 10.9 | 10.6 | 6.9 | 6.3 | | | Normal. |
| | " 13 | 57.2 | 5.1 | | 3.1 | 2.9 | | | Severe tetany, 4 days after operation. |
| 19 | " 11 | 52.1 | 10.6 | | 5.9 | 5.5 | | | Normal. |
| | " 12 | 32.0 | 6.9 | 6.1 | 3.2 | 2.2 | | | Severe tetany, 1 day after operation; dead. |
| 21 | " 17 | 45.3 | 11.4 | 11.1 | 5.9 | 5.2 | | | Normal. |
| | " 19 | 46.2 | 5.4 | 4.7 | 2.4 | 2.5 | | | Moderate tetany, 2 days after operation. |
| 22 | " 25 | 43.5 | 10.7 | 10.1 | 4.9 | 4.7 | 5.4 | 50.7 | Normal. |
| | May 2 | 44.9 | 6.0 | 5.6 | 3.7 | 2.7 | | | Severe tetany, 5 days after operation. |
| | " 5 | 45.7 | 5.9 | 5.7 | | | 4.6 | 78.0 | Mild tetany. |
| 29 | Nov. 28 | | 10.3 | 9.7 | | | 5.5 | 53.2 | Normal. |
| | Dec. 3 | | 6.4 | | | | 4.9 | 76.4 | No marked symptoms of tetany, 4 days after operation. |
| | " 4 | | 5.3 | 5.5 | | | 4.6 | 86.5 | Severe tetany, 5 days after operation. |
| 5 | Mar. 6 | 53.8 | 11.4 | 10.8 | 6.6 | 6.1 | 5.7 | 50.0 | Normal. |
| | Apr. 4 | 61.7 | 5.3 | 5.3 | 3.9 | 3.3 | | | Severe tetany, 13 days after operation. |
| | " 17 | 60.0 | 4.0 | 3.9 | 2.5 | 2.4 | 3 7 | 93.5 | Mild attack of tetany. Next 3 days total of 6 cc. parathormone injected in number of small doses. |
| | " 20 | 63.1 | 8.8 | 8.2 | 7.1 | | 4.9 | 52.0 | No symptoms of tetany. |
| | May 11 | 61.8 | 3.5 | 3.2 | | | | | |
| 20 | Apr. 14 | 50.6 | 10.1 | 9.9 | 5.8 | 5.1 | | | Normal. |
| | " 16 | 52.1 | 5.3 | 5.1 | 3.2 | 2.8 | | | Severe tetany, 2 days after operation. |

* C.S.F.Ca indicates cerebrospinal fluid calcium.

TABLE II—*Concluded.*

| Dog No. | Date. | Plasma volume. | Ca per 100 cc. | | | | | $\frac{\text{C.S.F.Ca}^*}{\text{Serum Ca}} \times 100.$ | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | | Serum. | Plasma. | Blood. | Blood (calculated). | Cerebrospinal fluid. | | |
| | *1928* | *per cent* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | | |
| 20 | Apr. 24 | 70.7 | 5.0 | 5.0 | 4.2 | 3.6 | 4.6 | 93.0 | Tetany controlled with small doses of parathormone; last injection 3 days previously, Apr. 21. |
| | " 25 | 71.7 | 10.2 | 10.0 | 7.3 | 7.3 | | | 3.5 cc. parathormone in 3 injections, also CaCl$_2$ solution by stomach tube. |
| 25 | May 14 | 50.2 | 10.9 | 10.3 | | | 5.6 | 51.7 | Normal. |
| | " 19 | 52.1 | 5.7 | 5.5 | | | 5.2 | 92.3 | 5 days after operation. Previous day a mild attack of tetany. |
| | " 22 | 58.2 | 11.0 | 9.9 | | | 5.0 | 45.8 | Severe attack of tetany day before. Injected 6 cc. parathormone in 4 doses, also 750 cc. milk by stomach tube. |
| | " 23 | | 8.4 | | | | 5.4 | 64.2 | |
| 30 | Dec. 5 | | 10.5 | 10.3 | | | 5.4 | 51.7 | Normal. |
| | " 6 | | 6.1 | 6.0 | | | 4.8 | 78.8 | Severe tetany, 1 day after operation. |
| | " 8 | | 16.7 | 15.5 | | | 5.4 | 34.7 | 8 cc. parathormone injected in 4 doses during previous 2 days; also 10 gm. mixture of CaCl$_2$ and lactate by stomach tube day before. |

plasma calcium dropped it invariably remained above the cerebrospinal fluid calcium level. Likewise, when the serum calcium level was raised by administering parathyroid hormone the normal ratio between cerebrospinal fluid calcium and serum calcium was rapidly restored provided the serum calcium was not built up above the normal level.[2] Otherwise, the cerebrospinal

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[2] We wish to acknowledge our indebtedness to Eli Lilly and to Parke, Davis and Company for their generosity in supplying us with their preparations of the parathyroid hormone. We also wish to express our thanks to Dr. M. Beber for his constant readiness to help us in the experiments.

fluid calcium failed to keep pace and the ratio between it and the serum calcium fell below the usual 50 ± per cent ratio, as was also the case in normal, unoperated dogs. This suggests that, though the cerebrospinal calcium may be dialyzed from the plasma, there must be some additional mechanism involved in governing its rise and fall, which is independent of the physicochemical condition of the ordinary membrane equilibrium.

### *Does Calcium Pass into the Blood Cells during Tetany?*

Our experiments were made on dogs which have been completely thyroparathyroidectomized. In an endeavor to explain the marked fall in the serum calcium in parathyroid tetany it has been suggested that calcium might pass from the serum into the red blood cells. At any rate, Cameron and Moorhouse's data, as one of us has shown previously (16), would suggest such a possibility, though these authors themselves do not regard such a transfer probable. Bär (1) actually maintains that this shift to the cells plays an important rôle in tetany, though we are unable to discover any basis for this in his data. Our own findings do not indicate such a transference of calcium, for the ten analyses we made on seven dogs point to the presence of only a slight and inappreciable amount of calcium in the red cells of the parathyroidectomized animals, from 0.1 to 1.0 mg. per cent with an average of 0.48 mg. per cent. It will be noted that these figures are of about the same magnitude as those we obtained for the calcium in the cells of normal dogs. The findings of Cameron and Moorhouse on the whole blood calcium, if compared with the concentrations which they report for plasma would indicate that the cells contain between 0.5 and 2.9 mg., with an average of 1.2 mg. per cent. We would not, however, draw from their data any conclusions regarding the calcium content of the red corpuscles in parathyroid tetany on account of the marked differences which they record between serum and plasma calcium concentrations.

### *Does Blood Contain a Slowly Dissociating Organic Calcium Compound?*

Cameron and Moorhouse believe that in normal plasma the calcium is present in an inorganic, diffusible form to the extent of

53 per cent, the rest being a non-diffusible specific organic compound. The parathyroid hormone is thought to be this specific organic substance or that which controls its formation in the organism. This organic calcium compound is regarded as very slightly dissociable, holding through a series of interlocked equilibria a definite amount of inorganic calcium in the plasma. This organic calcium is also supposed to be somehow taken up by the clot following parathyroidectomy, thus causing the large discrepancy between the serum and plasma calcium concentration. We have already shown that there is apparently no basis of fact to support this contention.

By rapidly centrifuging oxalated plasma they believe, however, to have been able to demonstrate the presence of such a slowly dissociating organic calcium compound. According to their data the calcium determined immediately in samples of oxalated plasma from two normal dogs was 2.5 to 3.3 mg. per cent. Analyzed about 2 to 4 hours later the calcium in different samples was from 0.5 to 1.6 mg. per cent. Cameron and Moorhouse assert that these results support their hypothesis of the existence of an organic calcium compound slowly dissociating after removal of calcium ions. One is utterly at a loss to understand how this interpretation fits their findings, since they report higher values immediately after centrifuging than several hours later, whereas the reverse might be expected under the circumstances. Besides, the analyses of such small quantities of calcium are entirely unreliable, the error being so large as to completely invalidate the importance of the experiments.

Studying the course of precipitation of serum calcium upon the addition of oxalate we found that within 7 minutes practically 90 per cent of the total calcium is precipitated. This is also true for a pure calcium chloride solution of the same calcium concentration. In fact, when one plots the course of the calcium precipitation, the curves for the serum and for the pure calcium chloride solution can be superimposed without appreciable discrepancy between the two. Now, if one takes into consideration that an oxalate concentration of about 0.025 molar must be used to prevent clotting, whereas we have found that even a 1/320 molar concentration suffices to precipitate over 90 per cent of all the calcium in 7 minutes, Cameron and Moorhouse must have lost by far the largest part of the blood calcium by the time they have separated the plasma. They say that the blood was centrifuged "immediately," but even so the time intervening between the addition of the oxalate and removing the plasma must have been not less than 10 minutes. In that time the calcium present in the blood would be already at least 90 per cent precipitated. The recovery of minute amounts of calcium upon further addition of oxalate to the plasma is very uncertain. An amount of 0.5 mg. of Ca would require a titration with only 0.1 cc. of 0.005 N $KMnO_4$. In view of these considerations the whole conception developed by Cameron and Moorhouse with regard to the different behavior of calcium in the blood of normal and of parathyroidectomized animals appears extremely uncritical.

Greenwald (11) also concluded that the evidence offered by Cameron and Moorhouse for the presence of an organic calcium compound especially related to parathyroid function was not satisfactory. He attempted, however, to interpret, without seriously questioning, their experimental findings. Since we have actually shown that the red corpuscles of parathyroidectomized dogs, like those of normal animals, contain no appreciable amounts of calcium, if indeed any, his explanation loses its significance. Greenwald's own conception of the organic calcium compound of serum which presumably is of the nature of a calcium citrate is most stimulating but as yet mainly circumstantial evidence and good logical reasoning are its only support.

### *D. Intravenous Injections of Calcium Salts.*

Considered as a dialysis phenomenon, it would seem that, if the amount of diffusible calcium in the blood were markedly increased and maintained at a high level for a number of hours, the spinal fluid calcium should also increase proportionately. In these experiments both normal and parathyroidectomized dogs were used. Generally, on the day previous to the experiment blood was taken by cardiac puncture and cerebrospinal fluid by inserting a needle into the cisterna magna, and the samples were analyzed for calcium. The next morning the animal was given 0.5 to 1 gr. of morphine and an hour later 0.22 gm. of chloretone per kilo of body weight by stomach tube. The animal was usually completely anesthetized in about half an hour. A modified Ringer solution was injected slowly through the vein of the leg. The solution used contained per liter the following constituents: 5 gm. of $CaCl_2$, 5 gm. of NaCl, 0.42 gm. of KCl, 0.2 gm. of $NaHCO_3$, and enough $CO_2$ to keep all the calcium in solution. It was run in through a spiral glass tube immersed in a warm water bath so that the solution entering the vein was practically at body temperature. The flow was so adjusted that about 3 cc. were admitted per minute. The injection was continued usually about 6 hours during which time 1 liter of the solution was injected. Samples of blood and spinal fluid were taken approximately ½ hour after the injection was stopped. Since Cameron and Moorhouse suggest that the function of the parathyroid hormone may be to convert diffusible calcium into a non-diffusible organic form, we wished to determine whether there was any marked difference between the increase in spinal fluid calcium after the intravenous injection of the $CaCl_2$ solution in animals with a normal amount of the hormone and in those with the hormone lacking. In the few experiments which we carried out it is apparent that the increases in spinal fluid calcium were no greater in the parathyroidectomized dogs than in the normal dog; in fact, the largest increase was observed in a normal dog (Dog 41). While the increase in serum calcium amounted to from 5 to 18 mg. per cent the rise in spinal fluid calcium was only 0.7 to 2.7 mg. per cent.

These experiments show that the cerebrospinal fluid calcium is less subject to variations than the blood calcium. In the parathyroidectomized animals we observed that a relatively small diminution in the calcium of the cerebrospinal fluid is associated with the attacks of tetany. But by raising the blood calcium level with parathyroid hormone in operated dogs the cerebrospinal fluid calcium was not increased beyond its normal level, while by administering calcium and hormone together to normal dogs the spinal fluid calcium increased at most about 20 per cent, and generally very much less, although the serum calcium was increased 50 per cent or more. Even raising the serum calcium to very high levels (two or nearly three times its initial value) by means of intravenous injections of calcium and maintaining such high levels for several hours has not caused generally a much greater rise in the spinal fluid calcium, though in a few instances under a very greatly increased calcium pressure in the serum an

TABLE III.

*Intravenous Injections of Calcium.*

| Dog No. | Date. | Ca per 100 cc. | | $\frac{\text{C.S.F.Ca*}}{\text{Serum Ca}} \times 100.$ | Remarks. |
|---|---|---|---|---|---|
| | | Serum. | Cerebrospinal fluid. | | |
| | *1929* | *mg.* | *mg.* | | |
| 34 | Feb. 9 | 10.9 | 5.4 | 49.3 | Normal. |
| | " 11 | 26.4 | 6.6 | 25.1 | Injected Ca for 6 hrs. |
| 36 | " 15 | 12.6 | 5.2 | 41.8 | Normal. |
| | " 15 | 20.7 | 6.1 | 29.9 | Injected Ca for 6½ hrs. |
| 40 | Mar. 6 | 10.6 | 5.9 | 56.0 | Normal. |
| | " 7 | 20.2 | 7.7 | 38.1 | Injected Ca for 6 hrs. |
| 41 | " 13 | 11.4 | 5.6 | 46.6 | Normal. |
| | " 13 | 29.6 | 8.3 | 28.0 | Injected Ca for 6 hrs. |
| 43 | " 13 | 13.3 | 5.5 | 41.6 | Normal. |
| | " 21 | 25.4 | 6.4 | 25.2 | Injected Ca for 6 hrs. |
| 46 | " 22 | 10.9 | 5.5 | 50.4 | Normal. |
| | " 22 | 16.8 | 6.5 | 38.6 | Injected Ca for 6 hrs. |
| 48 | " 28 | 13.4 | 6.6 | 49.3 | Normal. |
| | " 28 | 23.3 | 6.9 | 29.6 | Injected Ca for 6 hrs. |
| | *1930* | | | | |
| 52 | Feb. 24 | 11.7 | 5.7 | 48.7 | Normal. |
| | " 24 | 15.0 | 6.3 | 42.0 | Injected Ca for 6 hrs. |
| | Mar. 11 | 5.9 | 5.2 | 88.3 | Parathyroidectomized 6 days ago. |
| | " 11 | 14.0 | 5.5 | 39.2 | After injecting Ca for 6 hrs. |
| | *1929* | | | | |
| 37 | Feb. 19 | 10.9 | 5.5 | 50.5 | Normal. |
| | " 25 | 18.8 | 6.2 | 32.7 | 2 days after parathyroidectomy, injected Ca for 6½ hrs. |
| | Mar. 1 | 8.2 | 4.6 | 56.5 | Parathyroidectomized; before injection of Ca. |
| | " 1 | 18.2 | 6.0 | 33.0 | After injecting Ca for 6½ hrs. |
| 39 | Feb. 5 | 11.3 | 5.5 | 48.5 | Normal. |
| | " 8 | 6.8 | 5.1 | 75.0 | Parathyroidectomized 3 days ago. Tetany attack. |
| | " 8 | 19.3 | 5.8 | 30.4 | Same after injecting Ca for 6 hrs. |

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* C.S.F.Ca indicates cerebrospinal fluid calcium.

increase in the cerebrospinal fluid calcium as high as 50 per cent was observed (Dogs 40 and 41, Table III). On the other hand, practically no difference was observed in the behavior of the cerebrospinal fluid calcium of normal and of parathyroidectomized dogs. This again indicates that the cerebrospinal fluid calcium level must be regulated by other factors besides physicochemical conditions alone. Possibly the rate at which the nervous tissue takes up the calcium from the fluid determines its ultimate level. This is a point very difficult to decide experimentally. We have attempted to study the rate of disappearance of calcium injected directly into the spinal cavity. The injected calcium disappears very rapidly but, of course, this does not prove that it might not have diffused back into the blood, instead of being taken up by the nervous tissue.[3]

### *Diffusible and Non-Diffusible Calcium.*

Several studies have been made of the diffusible and non-diffusible calcium of the serum and plasma of parathyroidectomized dogs in an endeavor to determine whether the striking drop in calcium concentration is reflected in both fractions or in only one. Moritz (17), in 1925, studied by the method of ultrafiltration the serum calcium of seven cases of hypocalcemia in rabbits following parathyroidectomy. His data indicate that in six of these the *diffusible calcium fell more rapidly* than the "colloidal," and in four which recovered from the attack the original ratio of diffusible to non-diffusible calcium tended to become reestablished as the total serum calcium rose. From a study of four cases of experimental tetany in dogs, von Meysenbug and McCann (15) state that, according to their findings, by the method of compensation dialysis, the diffusible calcium ranged from 58

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[3] Since this was written, Hertz reported (*Biochem. Z.*, **217**, 337 (1930)) that the ultrafiltrable Ca of the serum after parathyroidectomy, though only half as great as in normal dogs, shows a relative increase from 42 to 55 to 53 to 63 per cent of the total Ca. Under the influence of parathormone the ultrafiltrable fraction is markedly increased. Comparing the cerebrospinal fluid Ca with the ultrafiltrable Ca of the serum, Hertz finds that in the normal dog the two values are in sufficiently close agreement to justify the assumption that the former represents the dialyzable portion. This approximation of the cerebrospinal fluid Ca and the ultrafiltrable serum Ca, however, disappears in dogs developing tetany.

to 71 per cent with a total serum calcium between 6.1 and 8.4 mg. per cent. It would thus appear that the relative proportion of diffusible and non-diffusible calcium remained constant. Reed (20), using the method of ultrafiltration, determined the diffusible plasma calcium in parathyroidectomized dogs and his results indicate that certain individuals undergo a pronounced diminution of one fraction or the other. In his normal animals the diffusible calcium was between 40 and 70 per cent of the total. The maximum range in any one animal on successive days was 39 to 52 per cent, but usually the variation was within the experimental error of ± 4 per cent. He states that in parathyroid tetany there is first a decrease in the diffusible fraction of calcium which through a shift in equilibrium results in a transfer of non-diffusible calcium and a consequent diminution of this fraction. He thinks that this would explain the variability in the relative proportion of these two fractions, depending on the stage in the process of transfer in which the observation was made. Cruickshank (7) found that the diffusible calcium of the blood serum in severe parathyroid tetany amounted to 94 per cent of the total and concluded that the most rapid reduction occurred in the non-diffusible calcium fraction.

In view of this lack of agreement one cannot draw any definite conclusions regarding the proportions of diffusible and non-diffusible calcium in parathyroid tetany, except that the results of none of the investigators probably represent the true state of affairs.

Salvesen and Linder (21) found normal plasma proteins in two parathyroidectomized dogs while the serum calcium was, of course, diminished. They consider the non-diffusible calcium fraction to be bound to the plasma proteins, and, therefore, regard the decrease in serum calcium during tetany not as a primary fall in the protein-bound calcium but as a decrease in the "diffusible and ionized" fraction.

Cameron and Moorhouse in their studies of the calcium in the serum, plasma, and cerebrospinal fluid of dogs observed that the spinal fluid calcium was only slightly affected by parathyroidectomy instead of showing a fall in calcium parallel to that occurring in the serum or tending to disappear, as might be expected from Salvesen's conclusions.

We attempted to study this problem, avoiding the shortcomings of the usual method of compensation dialysis, by dialyzing the cerebrospinal fluid directly against the serum of the same animal. For this purpose we employed the Schleicher and Schüll dialyzing shells No. 579, placing a measured volume of the fresh serum inside and a similar volume of the cerebrospinal fluid obtained at the same time as the blood on the outside, and adjusting the inside and outside levels. The dialysis apparatus was left in a refrigerator for a period of 20 to 22 hours, with occasional shaking. Both the serum and fluid were analyzed for calcium before and after the dialysis. The dialyzing thimbles were filled with distilled water for exactly 10 minutes, then emptied and dried in an inverted position for 30 minutes, before being used. By adhering to this technique rigidly we obtained results of remarkable uniformity, although even under these experimental conditions it was impossible to prevent volume changes which are responsible for alterations in the calcium concentration. One can work by this procedure using a calcium chloride solution and water, and obtain theoretically correct results, but the situation is altogether different even when one dialyzes serum against cerebrospinal fluid, although the total salt concentration is practically the same on both sides of the membrane. By this procedure the conditions prevailing in the organism are more or less closely approached. In a series of experiments by this procedure we found that the cerebrospinal fluid practically always gained calcium during the dialysis. Following von Meysenbug's (15) method of calculation; namely,

$$\text{Per cent diffusible Ca} = \frac{(\text{Ca in dialyzate} \times 2) - \text{cerebrospinal fluid Ca}}{\text{Serum Ca}}$$

we obtained in a series of normal animals values between 61 and 63 per cent. This, of course, is a much greater ratio than that obtained directly from the study of the cerebrospinal fluid calcium. It is not hereby suggested that the higher value is the correct diffusible moiety, and all we wish to emphasize is that in our experience this is a reproducible value under the conditions of a definite technique. According to Cameron and Moorhouse (4) the parathyroid hormone either forms the non-diffusible organic calcium compound or promotes the formation of the specific organic substance which makes this compound. We, therefore, studied by the above procedure the changes in diffusible and non-diffusible fractions of calcium under different conditions. We studied the effect of injecting calcium salts into normal and parathyroidectomized dogs, also of injections of calcium together with parathyroid hormone, then dialyzing the dog serum against the cerebrospinal fluid. As the results in Table IV show, in the normal dog, following prolonged slow injection of calcium, the per cent of diffusible calcium obtained by dialyzing the serum against the spinal fluid is practically unchanged (57 to 61 per cent), and the addition of parathormone has no effect on this proportion (62 per cent). In parathyroidectomized dogs at the time they

TABLE IV.

*Dialysis Experiments.*

| Dog No. | Ca per 100 cc. | | | | Per cent diffusible Ca. | Remarks. |
|---|---|---|---|---|---|---|
| | Serum. | | Spinal fluid. | | | |
| | Before. | After. | Before. | After. | | |
| | *mg.* | *mg.* | *mg.* | *mg.* | | |
| 49 | 12.15 | 11.35 | 6.09 | 6.83 | 62 | Normal. |
| 44 | 24.50 | 19.26 | 6.52 | 10.85 | 62 | Normal dog. Ca injected intravenously for 6 hrs., and 1 cc. parathormone each hr. |
| 46 | 10.93 | 11.05 | 5.46 | 6.12 | 62 | Normal. |
| | 16.78 | 15.10 | 6.46 | 8 22 | 59.5 | After injecting Ca intravenously for 6 hrs. |
| 48 | 13.40 | 13.73 | 6.65 | 7.42 | 61 | Normal. |
| | 23.25 | 18.75 | 6.88 | 10.44 | 60 | After injecting Ca intravenously for 6 hrs. |
| 51 | 14.80 | 12.48 | 5.46 | 7.80 | 68 | Parathyroidectomized 5 days before; serum Ca dropped to 6 mg. per cent. Ca intravenously for 6 hrs. |
| 52 | 11.70 | 10.57 | 5.65 | 6.52 | 63 | Normal. |
| | 14.98 | 12.64 | 6 26 | 7.37 | 57 | After intravenous injection of Ca for 6 hrs. |
| | 5.90 | 6.52 | 5.18 | 4.04 | 49 | Parathyroidectomized. Mild tetany. |
| | 13.95 | 12.08 | 5.45 | 6.32 | 52 | After intravenous injection of Ca for 6 hrs. |

develop tetany one might anticipate that, since the specific organic substance necessary to form the non-diffusible calcium compound is absent, there would be a marked rise in the diffusible calcium upon the injection of a calcium salt. However, as the results show, a small increase in one case is fully compensated in another experiment by a corresponding decrease (52 and 68 per cent respectively). We can conclude, therefore, from these experiments that the parathyroid hormone has apparently no relation to the formation of an organic non-diffusible calcium compound in the animal organism.

### SUMMARY.

The relation of cerebrospinal fluid calcium to the calcium of serum has been investigated in man and in the dog. It was found that in experimental jaundice there was no change either in the serum or in the cerebrospinal fluid calcium. The ingestion of calcium salts caused only a small rise in the serum calcium without materially affecting the calcium of the cerebrospinal fluid. If the feeding of calcium was accompanied by injections of parathyroid hormone the serum calcium was raised considerably and beyond the response of the cerebrospinal fluid calcium. Similarly when the serum calcium was greatly increased by means of a continuous intravenous injection of a modified Ringer solution containing a very large amount of $CaCl_2$, the cerebrospinal fluid calcium remained relatively low, and only in a few instances did it undergo a large absolute increase. This tendency of the cerebrospinal fluid calcium to maintain its level within narrow limits suggests that perhaps other factors besides those of the membrane equilibrium are operative in regulating it. The parathyroid hormone apparently has no effect on the distribution of the calcium between serum and cerebrospinal fluid because the changes upon intravenous injection of calcium were the same both in normal and parathyroidectomized animals at the time the latter have developed tetany.

The transfer of calcium to the red cells following parathyroidectomy, and the existence of a slowly dissociable organic calcium compound in serum are discussed and reasons are given for denying both.

By dialyzing serum against cerebrospinal fluid, an attempt has

been made to throw light upon the problem of the quantitative relationship between the two calcium compounds, namely the diffusible inorganic and the non-diffusible organic, and whether the formation of the latter is governed by the parathyroid hormone. These dialyses were made under normal conditions and after continuous injection of massive doses of calcium chloride, with or without the addition of parathormone, also on parathyroidectomized or on unoperated dogs. The evidence obtained from these experiments tends to show that the parathyroid hormone does not have any effect upon the distribution of the calcium of serum and cerebrospinal fluid.

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# THE DETERMINATION OF BLOOD UREA NITROGEN BY DIRECT NESSLERIZATION.

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The determination of urea nitrogen in blood filtrates is one of the most troublesome procedures encountered in the hospital laboratory.

When the method used is that of distillation as recommended by Folin it is often necessary to repeat determinations because the solution has sucked back or frothed over. The results obtained by aeration, unless very carefully checked, are too often low and the method takes too much time for use in emergency cases.

These difficulties have long been recognized and attempts have been made to overcome them by direct Nesslerization. The method proposed by Karr[1] is frequently used but is not satisfactory if a number of determinations must be made, as the solutions invariably become turbid. The attempt of Roe and Irish[2] to prevent turbidity by the use of calcium phosphate precipitation is only partially successful as the turbidity is not entirely due to traces of protein.

A method using direct Nesslerization would be of great value in the hospital laboratory if the formation of turbid solutions could be prevented.

It has long been recognized that colloidal solutions may be stabilized by the presence of a protective colloid. Folin[3] uses gum ghatti as the stabilizing agency to prevent the precipitation of Prussian blue in his micro sugar method. This gum is equally effective in maintaining crystal-clear solutions of the complex

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[1] Karr, W. G., *J. Lab. and Clin. Med.*, **9**, 3 (1924).

[2] Roe, J. H., and Irish, O. J., *J. Lab. and Clin. Med.*, **11**, 1087 (1926).

[3] Folin, O., *J. Biol. Chem.*, **77**, 421 (1928).

ammonium-mercuric iodide compound. The protection given by the gum is so good that even when tap water was used to make up the solutions as much as 1 mg. of ammonium nitrogen per 100 ml. could be Nesslerized without any trace of turbidity. Without the gum, solutions containing tap water invariably become turbid. With distilled water 1 ml. of 2 per cent gum ghatti[4] will permit the Nesslerization of solutions containing as much as 2.5 mg. of nitrogen in 100 ml. without the appearance of turbidity for several hours. By using more of the gum even greater concentrations of ammonium salts can be kept clear. 2 ml. of gum ghatti solution will protect 4 mg. per cent of nitrogen when Nesslerized with 30 ml. of Nessler's solution. When such a solution was read against a standard containing 1 mg. of nitrogen in 100 ml. the error was only 2 per cent. The standard was set at 20.0 mm. and a reading of 4.9 mm. was obtained. This was equivalent to 4.08 mg. of nitrogen.

It is necessary that the standard contain the same concentration of gum ghatti as the unknown because the presence of the gum decreases the intensity of the color about 10 per cent. Thus, when 0.5 mg. of nitrogen was Nesslerized with 10.0 ml. of Nessler's solution and made up to 100 ml. in the presence of 1 ml. of the gum ghatti solution it read 22.3 mm. against a similar solution without the gum set at 20.0 mm. This is only 90 per cent of the standard. That this decrease in the intensity of the color does not affect the accuracy of the method is shown in Table I. The solution containing 0.4 mg. of nitrogen was used as the standard, and all the solutions contained 1 ml. of 2 per cent gum ghatti solution and 10 ml. of Nessler's solution except Solution 13 which held 15 ml. of Nessler's solution. It will be seen that the relation between the concentration of nitrogen and color intensity is excellent from 0.1 mg. to 1.6 mg.

The presence of urease also causes a slight decrease in the intensity of the color and for this reason the standard must contain the same concentration of enzyme as the filtrate. In order to lessen the effect of the urease a more concentrated solution than is

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[4] 20 gm. of soluble gum ghatti are suspended on a copper gauze just below the surface of the liquid in a liter graduate filled with distilled water. After standing for 24 hours at room temperature the solution is filtered and is ready for use.

customary is used. This is prepared by shaking 15 gm. of jack bean meal, 10 gm. of permutit, and 100 ml. of 16 per cent alcohol in a shaking machine for ½ hour and then filtering through a folded filter. The solution, which is still turbid, is allowed to stand in a 100 ml. graduate in the ice box for 24 hours. The clear supernatant liquid is pipetted off and will keep in the ice box for about a week. 20 drops of this preparation when added to a standard containing 0.3 mg. of nitrogen and 1 ml. of the gum ghatti solution in a 100 ml. volumetric flask caused a loss in color amounting to 3 per cent.

TABLE I.

*Relation between Concentration of Ammonium Nitrogen and Color Intensity.*

| Solution No. | Ammonium sulfate N. | |
|---|---|---|
| | Amount present. | Amount found. |
| | *mg.* | *mg.* |
| 1 | 0.1 | 0.10 |
| 2 | 0.2 | 0.19 |
| 3 | 0.3 | 0.31 |
| 4 | 0.4 | 0.40 |
| 5 | 0.5 | 0.51 |
| 6 | 0.6 | 0.60 |
| 7 | 0.7 | 0.70 |
| 8 | 0.8 | 0.80 |
| 9 | 0.9 | 0.89 |
| 10 | 1.0 | 1.00 |
| 11 | 1.1 | 1.13 |
| 12 | 1.2 | 1.21 |
| 13 | 1.6 | 1.63 |

All solutions were read against Solution 4 taken as standard, set at 10.0 for Solution 1 and at 20.0 for the rest.

For the determination 2 drops of this preparation are used in the filtrate and 8 drops in the standard. One drop of this urease solution will completely digest 1.0 mg. of urea nitrogen, as is shown in Table II. Known amounts of a carefully purified urea solution were measured out and diluted to 5 ml., and then 2 drops of phosphate buffer and 1 or 5 drops of the urease were added. The solutions were allowed to digest in a water bath at 55° for 30 minutes. Each solution was read against a standard containing the same amount of nitrogen from ammonium sulfate, and the same concentration of urease and gum ghatti.

The recovery of added urea is shown in Table III. The amount of urea in 5 ml. of blood filtrate was determined by the new method before and after the addition of known amounts of urea. It will be seen that the added urea is recovered with a maximum loss of only 2 per cent.

The determination of the urea is made as follows: 5 ml. of blood filtrate, 2 drops of phosphate buffer, and 2 drops of concentrated urease solution are placed in a clean test-tube in a water bath at 55° for 30 minutes. The solution is then transferred to a urea tube graduated at 12.5 and 25.0 ml. and rinsed three times with a little distilled water so that the volume does not exceed 10.0 ml. Then 1 ml. of diluted gum ghatti solution, prepared by diluting the stock solution 1 to 8, is added and thoroughly mixed. Finally 1.5 ml. of Nessler's solution are added, the volume made up to 12.5 ml. with distilled water, and the solution again mixed. The standard is prepared by placing 3 ml. of ammonium sulfate solution containing 0.3 mg. of nitrogen in a 50.0 ml. volumetric

TABLE II.

*Digestion of Urea with Concentrated Urease.*

| Urease used. | Standard $(NH_4)_2SO_4$. | Urea present. | Urea found. |
|---|---|---|---|
| *drops* | *mg N* | *mg. N* | *mg. N* |
| 1 | 1.0 | 1.0 | 0.99 |
| 5 | 1.0 | 1.0 | 1.01 |
| 1 | 0.25 | 0.25 | 0.26 |
| 1 | 0.05 | 0.05 | 0.05 |
| 5 | 0.05 | 0.05 | 0.05 |

TABLE III.

*Recovery of Added Urea.*

| Blood urea N. | Urea added. | Urea found. | Recovery. |
|---|---|---|---|
| *mg. per 100 ml* | *mg. N* | *mg. N* | *per cent* |
| 18.2 | 100.0 | 116.0 | 98.8 |
| 10.0 | 200.0 | 212.0 | 101.0 |
| 13.4 | 20.0 | 33.0 | 98.0 |

flask and adding 8 drops of urease and 4 ml. of the diluted gum ghatti solution. The solution is then diluted to about 40.0 ml. and after it is mixed thoroughly, 6.0 ml. of Nessler's solution are added and the flask filled to the mark. It will be noted that the concentration of the solutions is twice that usually used. This was done to compensate for the decrease in the intensity of the color as the light yellow colors are rather difficult to read.

TABLE IV.

*Blood Urea Nitrogen. Comparison of Direct, Aeration, and Distillation Methods.*

| Aeration. | Direct method. | |
|---|---|---|
| | Standard plus urease. | Standard without urease. |
| *mg. per 100 ml.* | *mg. per 100 ml.* | *mg. per 100 ml.* |
| 11.0 | 11.1 | |
| 11.5 | 11.3 | |
| 12.8 | 12.5 | 12.0 |
| 14.2 | 14.2 | 14.1 |
| 15.6 | 15.9 | |
| 17.1 | 16.8 | 16.5 |
| 17.5 | 17.7 | 16.7 |
| 24.6 | 25.0 | 24.2 |
| 58.8 | 60.0 | 58.2 |
| Distillation. | | |
| 14.6 | 15.0 | |
| 17.8 | 17.8 | |
| 23.6 | 23.3 | |
| 83.0 | 84.0 | |
| 103.0 | 104.0 | |

The following experiment was performed to show that the blood filtrate contained nothing which would change the color of the Nessler's solution when added to a known amount of ammonium sulfate solution.

A solution containing 0.6 mg. of nitrogen and 1 ml. of 2 per cent gum ghatti solution was Nesslerized with 10 ml. of Nessler's solution and diluted to 100 ml. It was compared with a second solution containing the same substances and in addition 5 ml. of blood filtrate. No difference in the two solutions could be detected. When this experiment was repeated with only 0.3 mg.

of nitrogen a gain of 0.5 per cent in intensity was noted in the solution containing the blood filtrate.

The figures obtained by this method agree very well with the results obtained on the same filtrates by aeration or distillation. In Table IV there are given the results obtained by the various methods. The second column gives the results obtained when urease was added to the standard and the third column the results of reading the same solution against another standard not containing urease. The average loss is about 3 per cent, which is also the figure obtained when the standards are read against each other.

TABLE V.

*Comparison of the New Method and the Aeration Method for Urea N When Read against the Same Standard.*

| New method. | Aeration method. |
|---|---|
| *mg. per 100 ml.* | *mg. per 100 ml.* |
| 8.69 | 9.23 |
| 9.86 | 9.93 |
| 9.93 | 9.83 |
| 10.07 | 10.13 |
| 10.63 | 10.52 |
| 10.63 | 10.79 |
| 10.79 | 10.79 |
| 11.62 | 11.36 |
| 13.39 | 13.27 |
| 13.63 | 13.51 |
| 15.87 | 15.87 |
| 16.94 | 16.75 |
| 17.04 | 16.75 |
| 19.60 | 19.10 |
| 24.39 | 22.72 |
| 65.41 | 64.74 |
| 70.82 | 71.40 |
| 178.5 | 174.4 |

For very high concentrations of urea it is better to increase the strength of the standard to 1.0 mg. in 100 ml. and to make up the filtrate to the same volume. Both solutions should contain 8 ml. of diluted gum ghatti, 15.0 ml. of Nessler's solution, and 2 drops of urease. Bloods containing from 50.0 to 400.0 mg. of urea nitrogen can be read without the slightest difficulty against this standard.

The use of a tube graduated to both 12.5 and 25.0 ml. permits the dilution of filtrates which appear to be slightly high, by adding a solution containing 1 ml. of gum ghatti stock solution per 100 ml. The volume is first brought to about 20.0 ml. and, after the addition of another 1.5 ml. of Nessler's solution, made up to 25 ml. with the weak gum ghatti solution.

As the very intense red colors obtained from the more concentrated solutions are much easier to match than the yellowish colors of the weaker ones, better results can be obtained from the very high filtrates by setting the standard at 40.0.

By using the gum in the routine non-protein nitrogen determinations it is possible to read filtrates from bloods containing as high as 120 mg. against the routine standard. This should bring about a considerable saving in time now lost by the repetition of the determinations which become turbid from too much nitrogen.

The figures given in Table V were obtained by the use of 5 drops of the concentrated urease solution and were read against a standard not containing any urease. These figures are in excellent agreement with the results obtained from the same filtrates by aeration. This would seem to indicate that the aeration method as used in routine work gives figures that are about 3 per cent too low. For clinical work this error is negligible and would indicate that the addition of urease to the standard is a refinement that is not absolutely necessary.

The writer wishes to express his appreciation to Miss Ella Perkins of the chemical laboratory of Jefferson Hospital from whom these figures were obtained.

# FAT-SOLUBLE VITAMINS.

## XXX. THE ANTIRACHITIC VALUE OF COW'S MILK AS MODIFIED BY THE FEEDING OF IRRADIATED YEAST.*

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The idea that milk, both cow's and human, is insufficiently supplied with vitamin D to meet the requirements for good bone building, is widely accepted. The question may well be raised whether too much emphasis is not being placed on vitamin D as the calcifying agent, without keeping in mind that other corrective measures may well be looked for. However, the study of the factors which determine the vitamin D content of milk is certainly more than of academic interest.

We (1) have recently reviewed the literature bearing on the attempts which have been made to increase the vitamin D content of milk by irradiation of the animal. There appears no question but that the antirachitic potency of goats' milk can be increased by irradiation of the goat with a quartz mercury vapor lamp (2, 3). Hess and Gerstenberger and their coworkers (4, 5) have reported that human milk can likewise be improved by irradiation of the nursing mother. The data on cow's milk are, to our mind, far from convincing.

When one takes into consideration the different conditions under which experimental animals are kept, the variability in their supply of reserve stores of vitamin D, and the variable feeding

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techniques, together with the variation in the criteria used for the estimation of the severity of rickets, it is probably not to be wondered at that the results obtained in different laboratories fail to be concordant. Before the etiology of rickets can be understood the contention that cow's milk is more conducive to the production of rickets in the human than human milk needs to be settled. The Detroit investigators (6) have reported that human milk fed in amounts of 25, 30, or 40 cc. daily to rats failed to demonstrate the presence of the antirachitic factor. However, under the same carefully controlled conditions 30 cc. of cow's milk fed daily for 7 days induced marked healing of rachitic lesions in rats. Sherman and Stiebeling (7) have also reported recently that cow's milk contains considerable amounts of vitamin D. They state: "The large numbers of experiments here briefly reported afford extensive and convincing evidence in confirmation of the fact that cow's milk as ordinarily produced in this country contains important amounts of vitamin D." Outhouse, Macy, and Brekke (6) in commenting on the widely varying reports on the amount of vitamin D present in milk, remark: "It is impossible to explain the conflicting results of the studies reviewed above, since environmental factors surrounding the lactating individual and variations in experimental technique of feeding the milk play important rôles in the analysis of the results."

Relating to the effect of feed and sunlight on the antirachitic potency of milk, Luce (8) concluded from her experiments: "The diet of the cow therefore appears to be the main factor in determining the anti-rachitic value of her milk, for with a diet deficient in fat-soluble vitamins it has been found impossible, by the action of sunlight alone, to raise materially the anti-rachitic value of her milk." Chick and Roscoe (9) concluded that: "The anti-rachitic value of the milk (vitamin D content), on the other hand, was found to depend principally upon the degree of insolation of the cow." "At the same time, there was some indication that the diet of the cow, in consisting of fresh green fodder, was a contributory factor."

Many investigators have sought to determine whether the antirachitic potency of milk can be increased by feeding the lactating animal either during pregnancy alone or during pregnancy and lactation a substance potent in vitamin D, such as cod liver oil. McCollum and coworkers (10) tried to determine with rats whether vitamin D given as cod liver oil to the mother rat passes into the milk. When the rats were fed cod liver oil before mating, during pregnancy, and during the first 2 weeks of nursing, considerable protection against rickets to the young was afforded, but not so much as when the young were continued on the cod liver oil ration. When the cod liver oil was fed only during the 1st week of the nursing period, there was very little protection against rickets.

Hess and Weinstock (11) gave mother rats on a standard diet various amounts of cod liver oil for varying periods during pregnancy and lactation. They found that the young were not protected against rickets. In this connection Hess may be quoted as follows (12): "Attempts have geen made also to improve the quality of the milk in this respect by adding various amounts of cod-liver oil to the ration of the cow. Something has been accomplished by this means but not sufficient to indicate that this method will be serviceable in connection with infant feeding. Lesné and Vagliano[1] found that 500 gm. of cod-liver oil have to be added to the daily ration of cows to give antirachitic property to their milk. The effect on the milk of feeding irradiated ergosterol to the cow has not yet been tested."

Wagner and coworkers (13) found that cows fed for 7 months with large doses of cod liver oil produced milk with no appreciable corrective properties for rickets in infants.

Eufinger and coworkers (14) have reported that colostrum or milk obtained from healthy human mothers does not contain sufficient vitamin D to cure severe rickets in rats, but that it is possible to enrich these secretions with vitamin D by feeding irradiated ergosterol in the form of the pharmaceutical preparation vigantol. The transfer appears to be an immediate one because severely rachitic rats were completely cured by feeding either colostrum or milk from human mothers who had been taking vigantol for only a few days. Hart and coworkers (15) fed milking cows 8 ounces of cod liver oil daily. Special care was taken to accustom the animals to the cod liver oil feeding before putting them on the experimental ration. Apparently the cod liver oil was poorly absorbed, because feeding experiments with chicks in which the feces of these cows in the form of an ether extract

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[1] Lesné, E., and Vagliano, *Compt. rend. Soc. biol.*, **91**, 143 (1924); *Compt. rend. Acad.*, **179**, 539 (1924).

were used as part of the ration, showed that the vitamin D content of the feces from cows receiving cod liver oil was much higher in vitamin D. The potency of the milk was not studied.

The question of the rôle of the diet in furnishing vitamin D for the organism is apparently far from being a simple one. Not only may various constituents of the diet influence the assimilation of vitamin D, but vitamin D itself, as we now know it, may occur in various forms, which in turn are available in different degrees to different species. The result is that it is as difficult to generalize on the probable effect of the ingestion of vitamin D as it is to foretell the action of light acting on the organism directly (16).

Various isolated tests which we have made on the vitamin D content of milk from cows receiving cod liver oil convinced us that while the vitamin D might be slightly increased by such a procedure, the feeding of cod liver oil was certainly not to be looked upon as a practical measure for the improvement of milk. We accordingly cast about for other sources of vitamin D which might be used to advantage. Tests carried out with rats revealed to us that dried yeast suitably irradiated with ultra-violet radiations could be made antirachitic to the extent that not more than 1 mg. of yeast was necessary to furnish 1 rat unit of vitamin D. When it is taken into consideration that ordinary cod liver oil, as usually furnished to the trade, contains 1 rat unit of vitamin D in from 25 to 100 mg. of oil, the potency of the yeast can be readily appreciated. We have evidence that the vitamin D in this yeast occurs in at least two different forms. Of their relative availability to the cow, we have no knowledge; yet the high total potency suggested that it might be well worth the attempt to determine whether or not it could be used for experimental purposes with this species. At the same time and as part of the experiment, we determined the effect of the feeding of this yeast on the calcium balance. Results on the latter have already been published (17). In this paper, we shall concern ourselves with the data bearing on the effect of yeast on the antirachitic potency of milk.

## EXPERIMENTAL.

The experiments here reported were performed during the last 2 months of 1928 and the first 4 months of 1929. They were purposely carried out in winter, to minimize the possible effect of sunlight. Exposure of both animals and ration to sunlight was purposely prevented, except in the cases of certain control animals which were taken as representative of herd conditions. All the cows except the general herd were Holsteins. They were in the 4th to the 9th month of lactation. Cows 1 and 2 received a daily basal ration of 10 pounds of alfalfa, 25 pounds corn silage, and 1 pound of a grain mixture for approximately each 3 pounds of milk produced. The grain ration was a mixture, composed of yellow corn meal 59 per cent, wheat bran 25, oil meal 15, and salt 1. Both cows received a supplement of 200 gm. of non-irradiated yeast for the first 4 weeks of the experiment. This constituted the preliminary period, and it was followed by the irradiated yeast period during which the non-irradiated yeast was displaced by irradiated yeast.

Cows 23, 30, and 32 received the same amount of roughage as Cows 1 and 2, and a grain mixture in the same proportions in relation to the milk produced which, however, consisted of yellow corn meal 35 parts, wheat bran 30, ground oats 30, oil meal 5, and salt 1. Cow 23 also received a supplement of 0.5 pounds of marl per day. With Cows 23 and 30, yeast was fed only during the irradiated yeast period. In other words, non-irradiated yeast was not fed in the preliminary feeding period. Cow 32 from which milk was obtained as representative of milk produced on a supplement of cod liver oil, had received 180 gm. of cod liver oil daily since October, 1926, and was continued on this supplement during the experiment. The rations on which the herd milk was produced are too varied in character for detailed description. In general, however, they consisted of mixed hay, corn silage, and a grain mixture. None of the cows in this group, however, received cod liver oil nor any special treatment, ration or otherwise, for the specific purpose of influencing the vitamin D content of the milk.

The yeast used for these experiments was finely ground, dried yeast which had been grown on molasses. It was kindly donated for these experiments by The Fleischmann Company. It was irradiated in quantities of 2 kilos at a time, spread out in a circle 6¾ feet in diameter at a distance of 2½ feet from the burners of a Cooper Hewitt quartz mercury vapor poultry treater or a Hanovia Alpine sun lamp. Both lamps were kindly loaned to us for experimental use by the manufacturers. The exposure of the yeast was continued for 2 hours. Every 30 minutes it was raked into a pile and then redistributed in order to secure as thorough exposure as possible. When fed to the cows it was thoroughly incorporated in a small portion of the grain mixture, in order to secure complete consumption.

To standardize the yeast for its antirachitic potency it was incorporated in 1, 5, or 10 mg. quantities in 50 gm. of our finely ground Ration 2965 (18). This consists of yellow corn 76, wheat gluten 20, calcium carbonate 3, and sodium chloride 1. The mixture of yeast and basal ration was fed to our standard rachitic rats with maintenance of consumption and weight records for a period of 10 days. When the 50 gm. had been completely consumed, unsupplemented Ration 2965 was fed for the rest of the 10 day period, the same records being kept as before. The rats were then killed and the distal ends of the radii and ulnæ examined for calcium deposits by the Johns Hopkins technique (19). The amount necessary to produce a continuous narrow line of calcium deposits in the majority of the rats on test is the unit which has been used by us for some time, primarily in the standardization of pharmaceutical products for describing the antirachitic strength of potent antirachitic agents. We have designated this amount of vitamin D as 1 rat unit. Our tests revealed that not more than a total of 1 mg.of the irradiated yeast was necessary to furnish this amount of vitamin D. On the other hand, we found that the cod liver oil which was fed during the time that our tests on the milk were being carried out required 50 mg. of the oil to produce the same effect. We had no check up on the potency of the oil which had been fed previously. The oil used can only be said to have been of high medicinal grade such as is generally available commercially.

Daily records were kept of milk production and composite samples were tested periodically for butter fat content. This will be discussed later. For our antirachitic tests with Cows 1 and 2, samples were taken after 4 weeks of feeding the non-irradiated and irradiated yeast respectively. The samples were measured out in 20 cc. quantities into 50 cc. Erlenmeyer flasks. These were stoppered with cotton and then kept frozen until used. In some cases where specifically designated, the milk was fed fresh. Before feeding the frozen samples, they were warmed, then mixed thoroughly, and cooled before the desired amounts were pipetted off.

The tests on the milk were carried out with the prophylactic technique. For each series there were used four litters of six rats each, with a litter representative in each group. In all of our work we have found it very important to have litter representatives distributed in this manner. It was this premise which made it necessary for us to feed frozen milk. We considered it far better to run the chance of encountering some destruction of vitamin D in the keeping process rather than to take the chance of introducing the large variables which are always encountered when animals are taken from different litters. The rats used were of our black and white piebald standard stock, weighing about 60 gm., at an age of 22 to 25 days. They were confined in individual screen cages on screen floors and weighed weekly. Distilled water and Ration 2965 were fed *ad libitum*. The milk was fed daily, measured out with a pipette into small porcelain dishes.

At the end of 5 weeks, the rats were killed and the femurs removed for determination of ash. They were dissected free from soft tissues and extracted with hot 95 per cent alcohol with frequent change of alcohol, in a Soxhlet apparatus, for 5 days. They were then dried and ashed. Ribs were removed for gross inspection of rachitic involvement, and the wrists for microscopic examination by means of the Johns Hopkins technique (19). Measurements of the width of the wrist bones and their metaphyses were made, since these dimensions increase in rickets and therefore could be used as an additional criterion of the comparative severity of the disease.

The first results are presented in Table I. The data of Table I were obtained by feeding 4 cc. of milk daily from Cows 1 and 2 during the two yeast periods. It can be said that after 3 weeks of milk feeding, all of the rats receiving milk obtained during the non-irradiated yeast period showed the enlarged wrists and a hopping, wobbling gait quite characteristic of rickets. The animals were very inactive; for the most part they remained in the back of their cages and their body tissues felt soft and relaxed. The rats receiving the same amount of milk obtained during the irradiated yeast period were apparently normal. They were very active and showed good muscular tone. What their general symptoms indicated is supported by the detailed data presented in Table I. Not only is the ash content from the feeding of the milk from the irradiated period much higher, but the percentage of ash is also higher. The radii and ulnæ as well as their metaphyses are narrower. This is important because, in general, it may be said that up to a certain point the more severe the rickets, the wider the bone.

### TABLE I.

*Antirachitic Value of Milk.*

*Bone Analyses of Rats on a Rachitogenic Ration Plus 4 Cc. of Milk Daily from Cows Which Received 200 Gm. of Non-Irradiated or Irradiated Yeast Daily.*

| Rat No. | | Condition of costochondral junctions. | Width of: | | | Ash in femora. | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Radii. | Ulnæ | Metaphyses | Average for rat. | Average for group. | Average for rat. | Average for group. |
| | | | *mm.* | *mm.* | *mm.* | *mg.* | *mg.* | *per cent* | *per cent* |
| 69 ♂ | Cow 1. Non-irradiated yeast period. | Very much enlarged. | 3.5 | 3.0 | 2.0 | 40.8 | | 32.4 | |
| 70 ♂ | | " " | 3.0 | 2.5 | 2 0 | 43.3 | | 34.3 | |
| 71 ♀ | | " " | 3.0 | 2.5 | 2.0 | 41.2 | | 34.3 | |
| 72 ♂ | | Slightly enlarged. | 3.0 | 2.5 | 1.0 | 81.9 | 51.8 | 47.0 | 37.0 |
| 93 ♂ | Cow 2. Non-irradiated yeast period. | Very much enlarged. | 3.0 | 2.0 | 2.0 | 38.1 | | 30.6 | |
| 94 ♀ | | Enlarged. | 3.0 | 3.0 | 1.5 | 48.8 | | 38.4 | |
| 95 ♀ | | Very much enlarged. | 3.0 | 2.0 | 1.5 | 41.0 | | 35.1 | |
| 96 ♀ | | " " | 3.0 | 2.5 | 2.0 | 39.5 | 41.9 | 32.7 | 34.2 |
| 73 ♀ | Cow 1. Irradiated yeast period. | Normal. | 3.0 | 2 0 | <0.5 | 94.3 | | 53.7 | |
| 74 ♂ | | " | 3.0 | 2.0 | <0.5 | 84.8 | | 50.9 | |
| 75 ♂ | | " | 3.0 | 2.0 | <0.5 | 89.0 | | 49.6 | |
| 76 ♂ | | " | 2.5 | 2.0 | <0.5 | 114 6 | 95.7 | 52.5 | 51.7 |
| 97 ♀ | Cow 2. Irradiated yeast period. | Normal. | 2.5 | 2.0 | <0.5 | 90.1 | | 50.6 | |
| 98 ♂ | | " | 2.5 | 2.0 | <0.5 | 93.1 | | 51.1 | |
| 99 ♀ | | " | 2.5 | 2.0 | <0.5 | 99.8 | | 53.8 | |
| 100 ♀ | | " | 2.5 | 2.0 | <0.5 | 94.3 | 94.3 | 53.6 | 52.3 |

As the values for the percentage of ash on the supplement of 4 cc. of milk from the irradiated yeast feeding are approximately the maximum which can be obtained on Ration 2965 by the addition of vitamin D, we considered it of interest to run another series of rat tests in which various levels of milk were fed. The levels selected were 0.5, 1.0, and 2.0 cc. daily. They were compared with 4 cc. of herd milk and 2 and 4 cc. of milk from Cow 32, which was the cow receiving a supplement of 180 gm. of cod liver oil daily. In these experiments the milk was fed fresh.

The results of these experiments are presented in Table II. Even 2 cc. of irradiated yeast milk produced bone of maximum percentage ash content. With 1 cc. the percentage was somewhat lower, and with 0.5 cc. decidedly rachitic. The weights of ash, however, were in all cases less than on 4 cc., as shown on Table I. It is interesting to note that it required 4 cc. of herd milk to produce approximately the same quality of bone as that produced on a supplement of 0.5 cc. of irradiated yeast milk. Milk from the cow receiving cod liver oil was decidedly inferior, being little if any better than herd milk.

Further data on cod liver oil feeding are presented in Table III. Here the milk was taken from the same cow, namely Cow 32. It was compared at a 4 cc. level of intake with milk from Cow 30 fed at levels of 2 and 4 cc. daily. Cow 30, after a preliminary period on a ration free from yeast, received 50 gm. of irradiated yeast. It is again to be noted that the milk from the cow receiving cod liver oil was not superior to ordinary milk such as was produced by Cow 32 during the preperiod. 50 gm. of irradiated yeast daily, however, had a decided effect on the milk.

Table IV presents data on a still lower level of irradiated yeast; namely, 10 gm. daily. These were obtained with Cow 23 in comparison with Cow 32, receiving cod liver oil. At these levels no differentiating effect of any kind between the two sources of vitamin D was demonstrable.

Inasmuch as vitamin D is fat-soluble, it was deemed possible that better determinations of antirachitic potency might be made on butter fat obtained from the respective milks. In the first place, it was considered worth while in view of the possibility that vitamin D might be concentrated in the fat, and in the second place, the feeding of fat, unlike the feeding of milk, does

TABLE II.

*Antirachitic Value of Milk at Different Levels.*

*Bone Analyses of Rats on Various Amounts of Fresh Milk from Cows on Ordinary Rations or the Same Supplemented with Irradiated Yeast or Cod Liver Oil.*

| Rat No. | Supplement to rachitogenic ration. | Condition of costochondral junctions. | Width of: |  |  | Ash in femora. Average for group. |  |
|---|---|---|---|---|---|---|---|
|  |  |  | Radii. | Ulnæ. | Metaphyses. |  |  |
|  |  |  | *mm.* | *mm.* | *mm.* | *mg.* | *per cent* |
| 109–112 | 0.5 cc. milk daily from Cow 1 fed irradiated yeast (200 gm. daily). | Slightly enlarged. | 3.0–3.5 | 2 0–2.5 | 1.0–1.5 | 49.9 | 40.7 |
| 113–116 | 1 cc. same. | Normal to slightly enlarged. | 2.0–3.0 | 1.5–2.5 | <0.5–1.0 | 62.5 | 47.3 |
| 117–120 | 2 " " | " | 2.5 | 2.0 | <0.5 | 79.6 | 51.3 |
| 121–124 | 4 " herd milk daily. | Apparently normal to very much enlarged. | 3.0–4.0 | 2.0–3.0 | 0.5–1.5 | 54.2 | 41.7 |
| 125–128 | 2 " milk daily from Cow 32 fed cod liver oil (180 gm. daily). | Enlarged to very much enlarged. | 3.0–3.5 | 2.0–2.5 | 1.0–2.0 | 43.9 | 35.8 |
| 129–132 | 4 cc. same. | Apparently normal to very much enlarged. | 3.0–3.5 | 2.0–2.5 | 0.7–1.5 | 66.5 | 44.5 |

Each group except the first contained two males and two females; the first contained three males and one female.

TABLE III.

*Effect of 50 Gm. of Irradiated Yeast in Comparison with 180 Gm. of Cod Liver Oil on Antirachitic Potency of Milk.*

| Rat No. | Supplement to rachitogenic ration. | Condition of costochondral junctions. | Width of: | | | Ash in femora. Average for group. | |
|---|---|---|---|---|---|---|---|
| | | | Radii. | Ulnæ. | Metaphyses. | | |
| | | | *mm.* | *mm.* | *mm.* | *mg.* | *per cent* |
| 169–172 | 2 cc. milk daily from Cow 30 from preperiod. | Slight to medium enlargement. | 3.0–3.5 | 2.0 | 1.0–2.0 | 47.9 | 38.5 |
| 173–176 | 4 cc. same. | Medium enlargement. | 3.0 | 2.0–2.5 | 1.0–1.5 | 55.3 | 40.8 |
| 181–184 | 2 " fresh milk daily from Cow 30 from irradiated yeast period. | Slight enlargement. | 2.0–3.0 | 2.0–2.5 | 1.0–1.5 | 60.5 | 42.7 |
| 185–188 | 4 cc. same. | Apparently normal to slight enlargement. | 2.5–3.5 | 1.8–2.5 | 0.8–1.0 | 78.5 | 47.2 |
| 189–192 | 4 " fresh milk daily from Cow 32 receiving cod liver oil. | Medium enlargement. | 3.0–3.5 | 2.0–2.8 | 1.2–1.5 | 51.7 | 39.2 |

first wo groups contained two males and two females; the third and fifth, three males and one female; the fourth h s and one male.

TABLE IV.

*Effect of 10 Gm. of Irradiated Yeast in Comparison with 180 Gm. of Cod Liver Oil on Antirachitic Potency of 4 Cc. of Milk Daily.*

| Rat No. | | Condition of costochondral junctions. | Width of: | | | Ash in femora. Average for group. | |
|---|---|---|---|---|---|---|---|
| | | | Radii. | Ulnæ | Metaphyses. | | |
| | | | *mm.* | *mm.* | *mm.* | *mg.* | *per cent* |
| 197–200 | Cow 23. Preperiod. | Medium enlargement. | 3.0 | 2.0–2.5 | 1.0–1.2 | 53.1 | 40.5 |
| 209–212 | " 23. Irradiated yeast period. | Slight to medium enlargement. | 3.0–3.5 | 2.0–2.5 | 1.0–1.2 | 48.4 | 38.8 |
| 213–216 | Cow 32. Cod liver oil. | Enlarged to apparently normal. | 3.0–3.5 | 2.0–3.0 | 1.0–1.5 | 54.3 | 40.3 |

The second group contained three females and one male; the others two females and two males.

not disturb the calcium and phosphorus intake of the test animals. In anticipation of the possible use of butter fat for this work, we had collected cream from the various cows, usually toward the end of their experimental periods. This cream was churned and the butter fat separated from the butter by decantation and filtering at temperatures slightly above melting. The samples were then preserved in the refrigerator in Mason jars.

The results of feeding butter fat are shown in Tables V and VI. The butter fat was fed at 100 and 200 mg. daily; it was measured out into small porcelain dishes, at a fixed temperature slightly above melting, with a calibrated pipette. The basal ration and general technique used in these tests were the same as for the milk samples already described.

Table V shows the effects obtained after feeding the cow 200 gm. of yeast, and Table VI the effects resulting from the feeding of 10 and 50 gm. of yeast respectively in comparison with a feeding of 180 gm. of cod liver oil. 200 mg. of butter fat produced on a supplement of 50 gm. of irradiated yeast gave maximum calcification of bone. When the amount of yeast fed was reduced to 10 gm. daily, the resultant butter fat was much less calcifying, but it was still superior to butter fat produced on the unsupplemented ration. 180 gm. of cod liver oil apparently produced about the same results as 10 gm. of yeast. Butter fat feeding therefore was apparently able to reveal differences not possible with the feeding of milk itself.

The question naturally arises whether the vitamin D in yeast is more assimilable than in cod liver oil. Tests carried out on the feces from Cows 1 and 2 as well as another cow, No. 3, which was on the same ration, revealed that these feces carried large amounts of vitamin D (17); in fact, amounts of about the same order as those found present in the cod liver oil experiments previously reported (15). We have no data on the actual comparative availability. Our data, as a matter of fact, do not eliminate the possibility that cod liver oil might have produced an increase in vitamin D in the milk of the same order as yeast, if the same number of vitamin units had actually been given. As a matter of fact, actual tests revealed that the 180 gm. of cod liver oil given daily carried only one-third as many units of vitamin D as the 10 gm. of yeast.

TABLE V.

*Antirachitic Value of Butter Fat.*

*Bone Analyses of Rats on Mixed Butter Fat from Cows 1 and 2 Both of Which Received 200 Gm. of Non-Irradiated or Irradiated Yeast Daily.*

| Rat No. | Supplement to rachitogenic ration. | Condition of costochondral junctions. | Width of: | | | Ash in femora. Average for group. | |
|---|---|---|---|---|---|---|---|
| | | | Radii. | Ulnæ. | Metaphyses. | | |
| | | | *mm.* | *mm.* | *mm.* | *mg.* | *per cent* |
| 77–80 | 100 mg. butter fat daily from non-irradiated yeast period. | Enlarged to enlarged with angulation. | 2.5–3.0 | 2.0–2.5 | 1.5–2.0 | 31.1 | 31.2 |
| 81–84 | 100 mg. butter fat daily from irradiated yeast period. | Normal. | 2.0–2.5 | 1.5–2.5 | <0.5 | 64.8 | 47.9 |
| 101–104 | 200 mg. butter fat daily from non-irradiated yeast period. | Enlarged to very much enlarged with angulation. | 2.5–3.0 | 2.0–2.5 | 1.5–2.0 | 38.8 | 35.7 |
| 105–108 | 200 mg. butter fat daily from irradiated yeast period. | Normal. | 2.0–2.5 | 1.5 | <0.5 | 72.1 | 49.9 |

The first and fourth groups contained two males and two females; the second, three males and one female; the third, three females and one male.

TABLE VI.

*Effect of 10 Gm. or 50 Gm. of Irradiated Yeast in Comparison with 180 Gm. of Cod Liver Oil on Antirachitic Potency of Butter Fat from Cows 23, 30, and 32.*

| Rat No. | Supplement to rachitogenic ration. | Condition of costochondral junctions. | Width of: Radii. | Width of: Ulnæ. | Width of: Metaphyses. | Ash in femora. Average for group. | |
|---|---|---|---|---|---|---|---|
| | | | *mm.* | *mm.* | *mm.* | *mg.* | *per cent* |
| 217–220 | 200 mg. butter fat daily from Cow 23 from preperiod. | Apparently normal to enlarged. | 3.0–3.8 | 2.0–3.0 | 1.0–1.5 | 46.4 | 38.6 |
| 221–224 | 100 mg. butter fat daily from Cow 23 from irradiated yeast period (10 gm. daily). | Slight to medium enlargement. | 2.5–3.5 | 2.0–2.2 | 0.8–1.2 | 46.0 | 38.5 |
| 225–228 | 200 mg. same. | Apparently normal to slight enlargement. | 2.5–3.4 | 2.0–2.5 | 0.9–1.0 | 53.6 | 43.5 |
| 229–232 | 200 " butter fat daily from Cow 30 from preperiod. | Slight to medium enlargement. | 2.7–3.2 | 2.0–2.5 | 0.9–1.2 | 48.3 | 40.8 |
| 233–236 | 200 mg. butter fat daily from Cow 30 from irradiated yeast period (50 gm. daily). | Apparently normal. | 2.2–2.8 | 1.5–2.0 | 0.5 | 73.8 | 51.7 |
| 237–240 | 200 mg. butter fat daily from Cow 32 receiving cod liver oil (180 gm. daily). | Slight to medium enlargement. | 3.0–3.4 | 2.0–2.4 | 0.8–1.0 | 60.1 | 46.2 |

All except the fourth group contained two males and two females; the fourth contained three females and one male.

In the practical feeding of cod liver oil there is, however, another factor which must be considered. Drummond and co-workers (20) in studying the effect of cod liver oil on vitamin A in milk found that, "The administration of cod liver oil caused no appreciable change in the yield of milk but the higher doses appeared to cause a noticeable drop in the percentage of fat." In a second paper Golding *et al.* (21) reported that "daily addition of 2 oz. of cod liver oil to this ration did not significantly depress the percentage of milk fat nor did it raise the vitamin D of the butter to any appreciable extent. Higher doses of cod liver oil depressed the percentage of milk fat and raised the antirachitic potency of the butter." Golding (22) reported that the fat content of cow's milk can be reduced by feeding more than 4 to 6 ounces of cod liver oil per cow per day. He found no such fat reduction when the unsaponifiable fraction of cod liver oil, equivalent to 8 ounces of cod liver oil, was put into solution in peanut oil, nor when a commercial preparation of the vitamin fraction of cod liver oil was fed in the same way.

TABLE VII.

*Production of Milk and Butter Fat per Week on Rations Containing 200 Gm. of Yeast Daily.*

| Period. | Average milk production. | | | Average butter fat content in milk. | | | Average weight of fat. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Cow 1. | Cow 2. | Cow 3. | Cow 1. | Cow 2. | Cow 3. | Cow 1. | Cow 2 | Cow 3. |
| | *lbs.* | *lbs.* | *lbs* | *per cent* | *per cent* | *per cent* | *lbs.* | *lbs.* | *lbs.* |
| 3 wks. before irradiated yeast was fed. | 288.0 | 354.5 | 273.5 | 3.2 | 3.7 | 3.0 | 9.21 | 13.23 | 8.3 |
| 8–10th wks. after irradiated yeast feeding was started. | 249.0 | 292.8 | 237.6 | 3.2 | 3.8 | 3.2 | 7.97 | 10.69 | 7.52 |
| 33–35th wks. after irradiated yeast feeding was started. | 176.2 | 233.8 | 201.1 | 3.3 | 3.7 | 3.1 | 5.75 | 8.57 | 6.17 |

Data similar to the above have been obtained in this laboratory in connection with other experiments which have entirely confirmed the aforementioned citations. Table VII gives the production of milk and butter fat at stated periods from our cows which received

200 gm. of irradiated yeast daily. It will be noted from the records that there resulted no reduction in the percentage of fat, nor a rapid falling off in the amount of milk produced. As a matter of fact, the milk flow was remarkably well maintained. It is therefore evident that the depression in the butter fat content of milk resulting from the feeding of cod liver oil is not due to its vitamin D content *per se*.

Our observations on the increase of vitamin D in milk by the feeding of irradiated yeast do not stand isolated. Since our work was completed, Wachdel (23) has published data which lead him to claim that not only was the vitamin D content of the milk increased, but the milk yield as well. It seems reasonably safe, therefore, to conclude that irradiated yeast can be used as a satisfactory source of vitamin D for the enrichment of milk in this dietary essential.

## SUMMARY.

50 gm. of irradiated yeast fed to cows were found to increase the antirachitic potency of milk. Even 200 gm. of yeast did not lower the milk production nor did it decrease the butter fat content. When the amount of yeast was reduced to 10 gm. daily, a slight effect on the antirachitic potency of the butter fat was still noticeable. This effect was not clearly demonstrable on the milk. 180 gm. of cod liver oil produced similar results to 10 gm. of yeast. It appears that the feeding of a standardized irradiated yeast may be considered as a practical measure for the production of a milk of standard antirachitic potency. It remains to be demonstrated whether irradiated yeast can be effectively used for the same purpose in the human dietary.

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# THE CONDUCTANCE AND ACTIVITY COEFFICIENTS OF GLUTAMIC AND ASPARTIC ACIDS AND THEIR MONOSODIUM SALTS.*

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In this paper the results of the measurements of the conductivity at 0° and of the freezing point of dilute aqueous solutions of aspartic and glutamic acids and their monosodium salts are reported. The results are interpreted with the aid of modern thermodynamic methods. It is shown that we must assume the existence of neutral micelles[1] to explain the results obtained with the free amino acids while the data for the salts may be explained on the assumption of ionic micelles alone. The high degree of ionization of the salts in dilute solutions makes it improbable that neutral micelles exist to an appreciable extent.

In recent years the dissociation constants of most of the naturally occurring amino acids have become available (1). Data for the activity coefficients of these substances have not yet been obtained. Studies on the activity coefficients of soap solutions (2), alkali silicate solutions (3), and certain weak electrolytes (4) have been reported. Adair (5) and Stadie and Sunderman (6) have reported activity data dealing with hemoglobin.

## *Apparatus and Experimental Methods.*

The procedure consisted in determining the freezing point depression of dilute solutions of the amino acids or their monosodium salts by means of a modification of the differential method

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[1] We have used the term micelle to denote an aggregation of molecules or of ions which in a solution act as a unit. The terms micelle or aggregate are used interchangeably.

described by Adams (7). The essential features are: (*a*) thorough mixing of the solution with a large amount of chipped ice, (*b*) determination with a multiple junction thermoelement and sensitive potentiometer of the difference between the equilibrium temperatures of ice solution of amino acid and ice water, (*c*) analysis of the equilibrium solution by means of a highly sensitive conductivity apparatus.

The equipment used consisted of the identical instruments used by Randall and Vanselow (8) and later improved by Randall and Scott (9). The freezing point assembly needs no further description except to add that the thermocouple was rechecked against melting ice and melting mercury. Exact agreement with previous calibration was obtained. The source of the 1000 cycle current was a modified Bell telephone circuit with a two bulb audion oscillator and a two bulb amplifier to intensify the circuit across the bridge. The method of Jones and Josephs (10) for balancing out the capacity effects was employed. The calibrations of the slide wire, the resistance box, and the potentiometer as determined by previous workers were accepted as correct. The procedure of Parker and Parker (11) was used in calibrating the conductivity cells by means of potassium chloride solutions. The Pyrex conductivity cells were maintained at 0° by immersion in an ice-water mixture kept in a gallon thermos jar. Efficient circulation was maintained by a revolving screw enclosed in a well.

In carrying out freezing point estimations, a mixture of washed chipped ice and conductivity water was placed in each of the two Dewar flasks. The flasks were evacuated for 15 minutes with stirring and air which had been passed over soda lime and phosphorus pentoxide was then admitted. After equilibrium had been attained and indicated by a very small and constant potential, portions of the chilled stock solution were added to the contents of one flask and after equilibrium had again been attained, the temperature difference between the contents of the two flasks was determined. A portion of the solution in equilibrium with ice was then used for conductivity measurement in order to estimate its concentration. To make certain at the beginning that the apparatus was functioning correctly the measurements of Randall and Vanselow (8) with hydrochloric acid were repeated and exact agreement with their results was obtained.

Glutamic acid was prepared from Ajinomoto according to the procedure of Schmidt and Foster (12). The aspartic acid was a Kahlbaum preparation. The amino acids were recrystallized three times from conductivity water. The products gave within experimental limits theoretical nitrogen values and were ash-free. The monosodium salts were prepared by adding calculated weights of the amino acids to approximately 0.15 M sodium hydroxide solutions. As soon as solution was complete the preparations were cooled and kept at 0° until used. The sodium hydroxide solution was prepared by distilling metallic sodium *in vacuo* and adding it to conductivity water in an atmosphere of nitrogen. The resulting solution was standardized against Bureau of Standards' benzoic acid, fused before use. The specific conductivity of the several samples of freshly redistilled water averaged about $0.4 \times 10^{-6}$ mhos.

In carrying out conductivity measurements solutions of each of the two amino acids and their monosodium salts were prepared by diluting the stock solutions, weight burettes for purposes of measurement being used. Conductivity readings were made at 30 minute intervals until the values obtained checked to 1 part in 50,000 which was the limiting sensitivity with the apparatus employed.

### *Conductivity Data.*

In order to evaluate the conductivity results in terms of molality in the freezing point measurements the logarithm of the molality was plotted against the logarithm of the conductance according to the procedure of Randall and Scott (9). The slope of the nearly straight line thus obtained was used in the formula

$$\log m = a \log c + b \qquad (1)$$

where $m$ = molality, $c$ = conductance, $b$ = a constant, and $a$ = the slope of the curve. A second plot of $\log c$ against $b$ was then made. This is very sensitive to changes in conductance. The graphs were used in the reverse order to estimate the concentration of amino acid in the freezing point measurements. In evaluating conductance values in terms of molality due regard was paid to the resistance of the lead wires, conductance of the water, and calibration of the measuring instruments. The lead

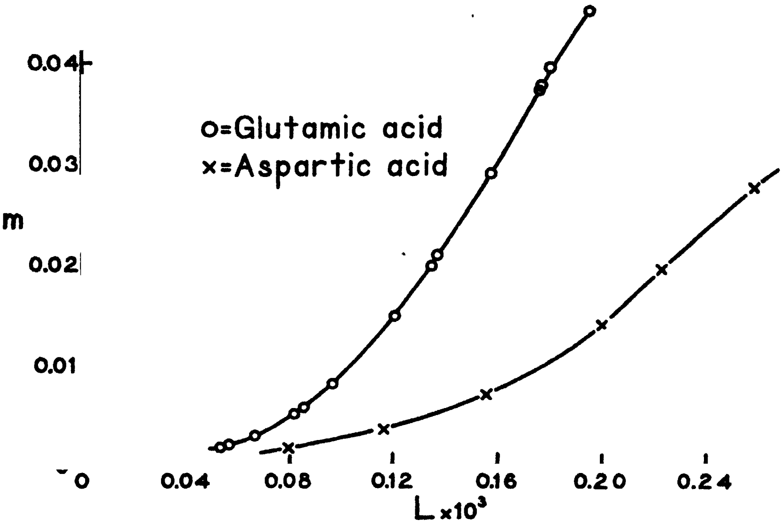

FIG. 1. Specific conductance of aspartic and glutamic acids at 0°.

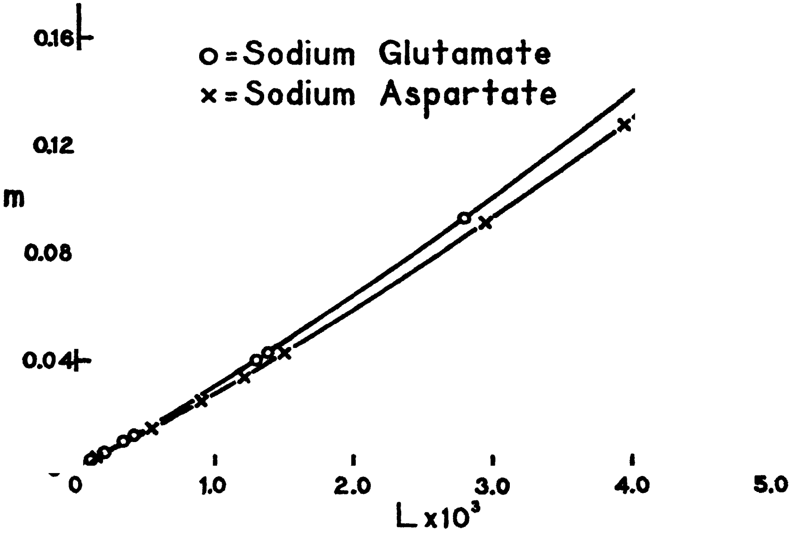

FIG. 2. Specific conductance of the solutions of the monosodium salts of aspartic and glutamic acids at 0°.

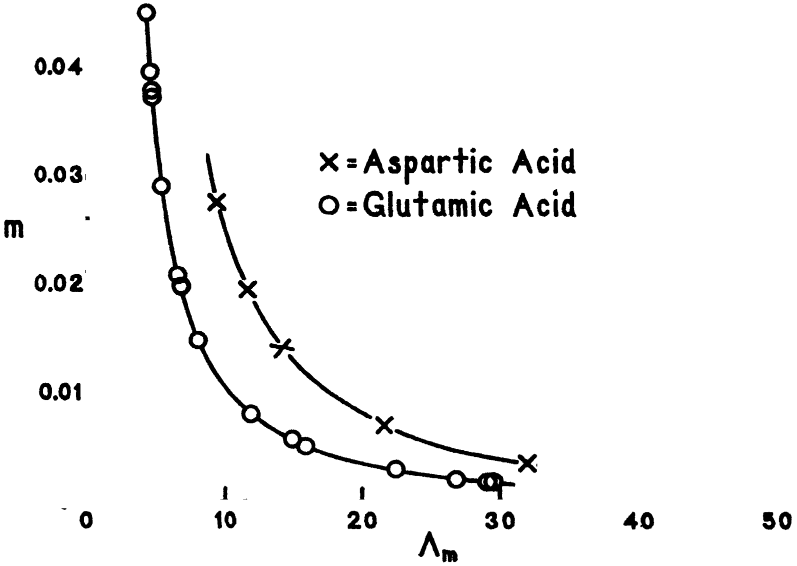

FIG. 3. Equivalent conductances of the solutions of aspartic and glutamic acids at 0°.

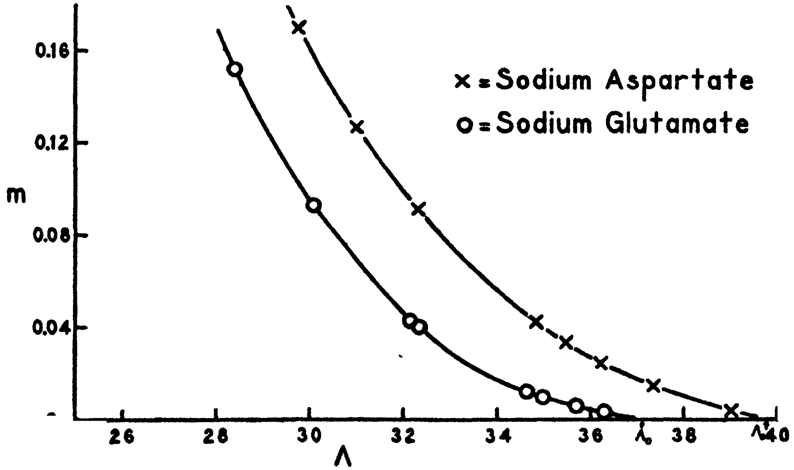

FIG. 4. Equivalent conductance of solutions of the monosodium salts of aspartic and glutamic acids.

TABLE I.

*Transference Numbers and Degree of Ionization of Aspartic and Glutamic Acids and their Monosodium Salts at 0°.**

| Substance analyzed. | $m$ | $\Lambda_{\text{aspartic acid}}$ | $\alpha$ | $\Lambda^{\circ}_{\overset{-}{\text{aspartate}}\text{ ion}}$ | $^{n}\text{H}^{+}$ | $n_{\overset{-}{\text{aspartate}}\text{ ion}}$ | $K_A \times 10^{4}$ |
|---|---|---|---|---|---|---|---|
| Aspartic acid. | 0 | 236 5 | 1.00 | 13 96 | 0 941 | 0 059 | 1.7† |
| | 0 005 | 26 7 | 0 113 | | | | 0.716 |
| | 0.01 | 17 6 | 0 074 | | | | 0 591 |
| | 0 02 | 11 5 | 0 048 | | | | 0.484 |
| | 0.03 | 9 0 | 0 038 | | | | 0.451 |
| | | $\Lambda_{\text{glutamic acid}}$ | | $\Lambda^{\circ}_{\overset{-}{\text{glutamate}}\text{ ion}}$ | | $n_{\overset{-}{\text{glutamate}}\text{ ion}}$ | $K_A \times 10^{5}$ |
| Glutamic acid. | 0 | 233 8 | 1 00 | 11 3 | 0.953 | 0.047 | 7.25† |
| | 0 005 | 16.0 | 0.068 | | | | 2.48 |
| | 0.01 | 10.3 | 0 044 | | | | 2 05 |
| | 0 02 | 6 7 | 0.029 | | | | 1.73 |
| | 0 03 | 5 3 | 0 022 | | | | 1.49 |
| | 0 05 | 4.0 | 0.017 | | | | 1.47 |
| | | $\Lambda_{\text{sodium aspartate}}$ | | $\Lambda^{\circ}_{\overset{-}{\text{aspartate}}\text{ ion}}$ | | $n_{\overset{-}{\text{aspartate}}\text{ ion}}$ | |
| Sodium aspartate. | 0 | 39 76 | 1.00 | 13.96 | 0.646 | 0 354 | |
| | 0.005 | 38 80 | 0 975 | | | | |
| | 0 01 | 37.94 | 0.954 | | | | |
| | 0.02 | 36 68 | 0.923 | | | | |
| | 0.05 | 34 37 | 0.865 | | | | |
| | 0 10 | 32 00 | 0.805 | | | | |

TABLE I—*Concluded.*

| **Substance analyzed.** | $m$ | $\Lambda_{\text{sodium glutamate}}$[*] | $\alpha$ | $\Lambda^{\circ}_{\overset{-}{\text{glutamate}}\text{ ion}}$ | $n_{H^+}$ | $n_{\overset{-}{\text{glutamate}}\text{ ion}}$ | $K_A \times 10^5$ |
|---|---|---|---|---|---|---|---|
| Sodium glutamate. | 0 | 37.10 | 1.00 | 11.3 | 0.696 | 0.304 | |
| | 0.005 | 35.90 | 0.968 | | | | |
| | 0 01 | 34.88 | 0.940 | | | | |
| | 0.02 | 33.80 | 0.911 | | | | |
| | 0.05 | 31 78 | 0.857 | | | | |
| | 0.10 | 29.86 | 0.805 | | | | |

[*] The following values taken from "International Critical Tables of Numerical Data, Physics, Chemistry and Technology," New York and London, 6, 232 and 241 (1926) were used in the calculations.

[†] Unpublished values at 0° by method of titration curves.

| Sodium chloride. | Hydrochloric acid. |
|---|---|
| $\Lambda^{\circ}_{c_{\text{NaCl}}} = 66\ 6$ mhos | $\Lambda^{\circ}_{\text{HCl}} = 262\ 4$ |
| $n_{\text{Na}^+} = 0\ 387$ | $n_{\text{H}^+} = 0.848$ |
| $\Lambda^{\circ}_{c_{\text{Na}^+}} = 25.8$ mhos | $\Lambda^{\circ}_{c_{\text{H}^+}} = 222\ 5$ |

In the above table $\Lambda = \dfrac{\text{specific conductance}}{\text{molality}}$

$$\Lambda_c \quad \frac{\text{specific conductance}}{\text{concentration}}$$

For all practical purposes $\Lambda^{\circ} = \Lambda^{\circ}_{c}$

wires were calibrated by filling the conductivity cells with mercury and determining the resistance of the circuit. The electromotive force readings were translated into temperature differences with the aid of the equation employed by Randall and Vanselow (8).

In Figs. 1 to 4 the specific conductance and the weight molal conductance of aspartic and glutamic acids and their monosodium salts are shown graphically. These need no further comment except to note the general similarity of the curves, which is to be expected in view of the close chemical relationship of these two amino acids.

By using known mobility data relating to hydrochloric acid and to sodium chloride (see foot-note to Table I) the equivalent conductance at infinite dilution and at 0° of hydrochloric acid and of sodium chloride was calculated to be 262.4 mhos and 66.6 mhos respectively. Since the transference numbers of the cations ($n_{H^+}$ and $n_{Na^+}$) under the same conditions are respectively 0.848 and 0.387, the ionic conductance at infinite dilution of the hydrogen ion and of the sodium ion are respectively 222.5 mhos and 25.8 mhos. As shown in Fig. 4 the equivalent conductance at infinite dilution of sodium aspartate and of sodium glutamate are respectively 39.76 mhos and 37.10 mhos. It follows that at infinite dilution and 0° the ionic conductances ($\Lambda°$) of the aspartate ion and of the glutamate ion are respectively 13.96 mhos and 11.30 mhos. The transference numbers under the same conditions are: $n_{\text{aspar}\overline{\text{a}}\text{te ion}} = 0.354$ and $n_{\text{glut}\overline{\text{a}}\text{mate ion}} = 0.304$. These

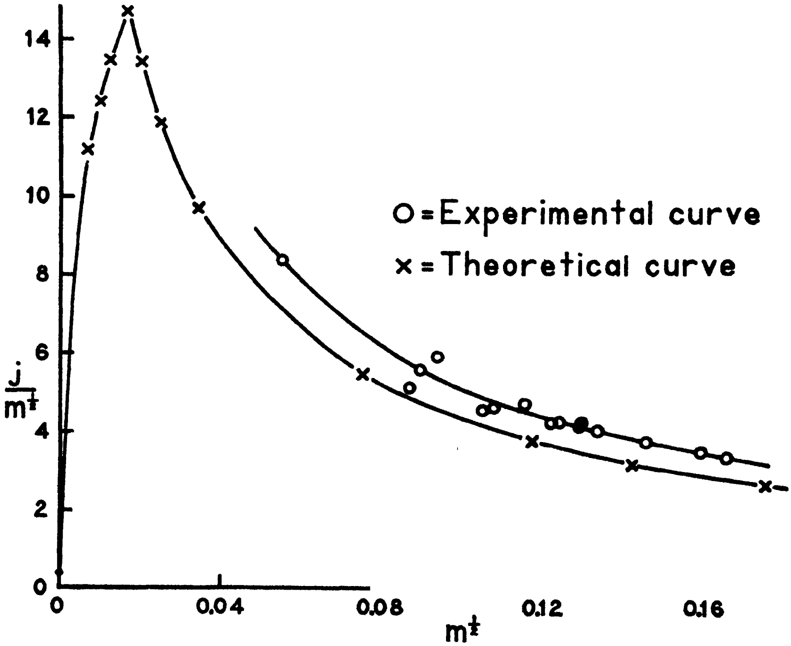

FIG. 5. Freezing point function for aspartic acid.

data are assembled in Table I. In the third column of Table I are given values for $\alpha$, the classical measure of the degree of ionization calculated from the formula $\alpha = \frac{\Lambda}{\Lambda^{\circ}}$. These values assume that the ionic mobilities are independent of the concentration. This is not strictly true in view of the micellation which we shall show takes place and varies with the concentration of the salts of the amino acids.

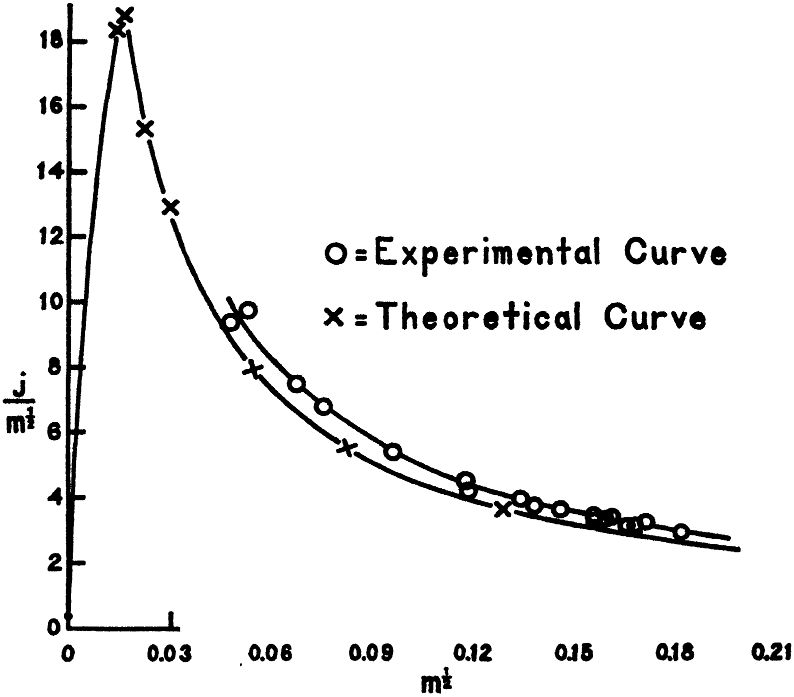

FIG. 6. Freezing point function for glutamic acid.

In the case of the free amino acids direct extrapolation of the equivalent conductance at infinite dilution is not possible but by adding the conductances of the two ions accurate values can be obtained and the degree of ionization and transference numbers can be calculated. These are given in Table I. It is evident that aspartic acid and glutamic acid are but slightly ionized even at

## TABLE II.

*Freezing Point Function.*

| Substance analyzed. | $m$ | $\theta$ | $\frac{\theta}{m}$ | $\frac{j}{m^{\frac{1}{2}}}$ |
|---|---|---|---|---|
| Aspartic acid. | 0.003034 | 0.006070 | 2.00 | 8.38 |
| | 0.007618 | 0.015596 | 2.05 | 5.15 |
| | 0.008019 | 0.014837 | 1.85 | 5.59 |
| | 0.008845 | 0.014533 | 1.64 | 5.93 |
| | 0.011044 | 0.021453 | 1.95 | 4.53 |
| | 0.012124 | 0.035475 | 1.93 | 3.78 |
| | 0.013402 | 0.022394 | 1.67 | 4.76 |
| | 0.014943 | 0.026407 | 1.77 | 4.28 |
| | 0.015760 | 0.021603 | 1.37 | 4.63 |
| | 0.016602 | 0.028804 | 1.73 | 4.14 |
| | 0.016780 | 0.028340 | 1.69 | 4.22 |
| | 0.017870 | 0.030642 | 1.71 | 4.02 |
| | 0.025301 | 0.041624 | 1.65 | 3.50 |
| | 0.027420 | 0.045457 | 1.66 | 3.34 |
| Glutamic acid. | 0.002301 | 0.004711 | 2.05 | 9.36 |
| | 0.002809 | 0.005029 | 1.79 | 9.77 |
| | 0.004549 | 0.008362 | 1.84 | 7.50 |
| | 0.005706 | 0.010270 | 1.80 | 6.83 |
| | 0.009158 | 0.016427 | 1.79 | 5.41 |
| | 0.013737 | 0.024060 | 1.75 | 4.51 |
| | 0.013839 | 0.024367 | 1.76 | 4.47 |
| | 0.014096 | 0.025978 | 1.84 | 4.23 |
| | 0.017930 | 0.031450 | 1.75 | 3.95 |
| | 0.019080 | 0.034318 | 1.80 | 3.73 |
| | 0.019588 | 0.034740 | 1.77 | 3.74 |
| | 0.021188 | 0.036823 | 1.74 | 3.64 |
| | 0.024198 | 0.041950 | 1.73 | 3.43 |
| | 0.024268 | 0.043534 | 1.79 | 3.32 |
| | 0.025274 | 0.043865 | 1.74 | 3.39 |
| | 0.027320 | 0.048702 | 1.78 | 3.15 |
| | 0.028200 | 0.049810 | 1.77 | 3.12 |
| | 0.029251 | 0.048723 | 1.67 | 3.23 |
| | 0.032947 | 0.057207 | 1.74 | 2.93 |
| Sodium aspartate. | 0.009332 | 0.032626 | 3.50 | 0.612 |
| | 0.009830 | 0.034012 | 3.46 | 0.695 |
| | 0.017748 | 0.060908 | 3.43 | 0.574 |
| | 0.018121 | 0.064696 | 3.57 | 0.289* |
| | 0.025200 | 0.086020 | 3.43 | 0.515 |
| | 0.026635 | 0.093540 | 3.51 | 0.335* |
| | 0.034385 | 0.118370 | 3.44 | 0.397 |
| | 0.042200 | 0.143840 | 3.40 | 0.402 |
| | 0.051056 | 0.174600 | 3.42 | 0.352 |

TABLE II—*Concluded.*

| Substance analysed. | $m$ | $\theta$ | $\frac{\theta}{m}$ | $\frac{j}{m^{\frac{1}{2}}}$ |
|---|---|---|---|---|
| Sodium glutamate. | 0.007503 | 0.026118 | 3.48 | 0.730 |
| | 0.008789 | 0.030242 | 3.43 | 0.789 |
| | 0.013675 | 0.046828 | 3.42 | 0.672 |
| | 0.019814 | 0.067309 | 3.40 | 0.611 |
| | 0.030900 | 0.104710 | 3.39 | 0.501 |
| | 0.046064 | 0.152575 | 3.32 | 0.507 |
| | 0.046448 | 0.156750 | 3.38 | 0.425 |
| | 0.056117 | 0.186630 | 3.33 | 0.442 |
| | 0 089275 | 0.298880 | 3.34 | 0.331 |
| | 0 097670 | 0.326710 | 3.34 | 0.319 |

* Equilibrium probably was not reached. Hence these values are probably not reliable.

low concentrations while the sodium salts are as highly ionized as most inorganic uniunivalent salts.

*Activity Coefficients of Aspartic and of Glutamic Acids.*

In Figs. 5 and 6 the values of $\frac{j}{m^{\frac{1}{2}}}$ (freezing point function) ((13) p. 326) are plotted against the values for $m^{\frac{1}{2}}$. All necessary data are given in Table II. Values for $j$ are obtained from the equation

$$j = 1 - \frac{\theta}{\lambda\, m\, \nu} \tag{2}$$

where $\nu$ = the number of ion molecules formed by the dissociation of a molecule, $\lambda$ = the molal lowering of the freezing point at infinite dilution (1.858), $m$ = the molality, and $\theta$ = the freezing point lowering. The ratio between a given molal lowering and the molal lowering at infinite dilution is given by $\frac{\theta}{\lambda\, m\, \nu}$. In calculating the experimental values for $j$ the assumption was made that the value for $\nu$ is equal to 2. This assumes that the ionization of the second carboxyl group is negligible as compared with the first. The free acids are thus assumed to be uniunivalent electrolytes.

In Figs. 5 and 6 values for a theoretical curve have also been plotted. The calculation of the theoretical curves demands some explanation. In developing the ideas of the activities of strong electrolytes, Lewis and Randall ((13) p. 326) showed that it was most convenient to define the activity of the undissociated part of an electrolyte as the geometrical mean of the activities of the ions and moreover the activity of the ion was defined as being equal to the stoichiometric molality of the ion when the concentration is very low.

It has recently been shown by Randall and Allen (14) that this same convention is convenient for the definition of the activity of the weak electrolytes.

On page 995 of Randall and Young's (15) article the values of $m$, $\theta$, and log $\gamma$ are given for hydrochloric acid. Using these data at various values of $m_i$, the concentration of the ionized part, Randall and Allen (14) have calculated $\gamma_i$, the mean activity coefficients of the ions of hydrochloric acid, $2\theta_i$, the freezing point lowering due to the ions, and $m_i^2\gamma_i^2$, which they show to be equal to $K_a m_u$, where $K_a$ is the dissociation constant of a weak acid and $m_u$ is the molality of the undissociated part of the weak acid. The sum of $m_i$ and $m_u$ gives the total molality $m$, and the total freezing point depression $\theta$ is the sum of $2\theta_i$ and $\lambda m_u$ since it was shown in the same article that at least for acetic acid $\theta_u = \lambda m_u$. The theoretical curves of Figs. 5 and 6 have been calculated in this way which involves the assumptions that over the concentration range studied the mean activity coefficient of the ions of a partially dissociated uniunivalent electrolyte is the same as that of a solution such as hydrochloric acid at the same ionic strength (*i.e.* $\frac{1}{2} \Sigma m_i Z_i^2$ where $m_i$ represents the molality of the $i^{\text{th}}$ kind of ion and $Z$ its valence) and that the ions and the undissociated molecules exert an independent effect on the freezing point. For very low concentrations use may be made of the relation:

$$\log \gamma = \log \gamma_{\pm} + \log \alpha \qquad (3)$$

where $\gamma$ is the stoichiometric activity coefficient defined with reference to an infinitely dilute solution, $\gamma_{\pm}$ is the activity coefficient of a strong acid, *e.g.* hydrochloric acid, at the same ionic strength, $\alpha$ is the degree of dissociation defined by the equation

$$K = \frac{(m\ \gamma_{\pm}\ \alpha)^2}{m - m\ \alpha} \qquad (4)$$

which is essentially the dissociation constant used by Sherrill and Noyes (16).

For aspartic acid, the value $2 \times 10^{-4}$ and for glutamic acid the value $6 \times 10^{-5}$ have been used for the primary acid dissociation constants at 25°.[2] In plotting our dotted theoretical curves, we have taken these same values at 0°. These have been taken from the tables of Kirk and Schmidt (1).

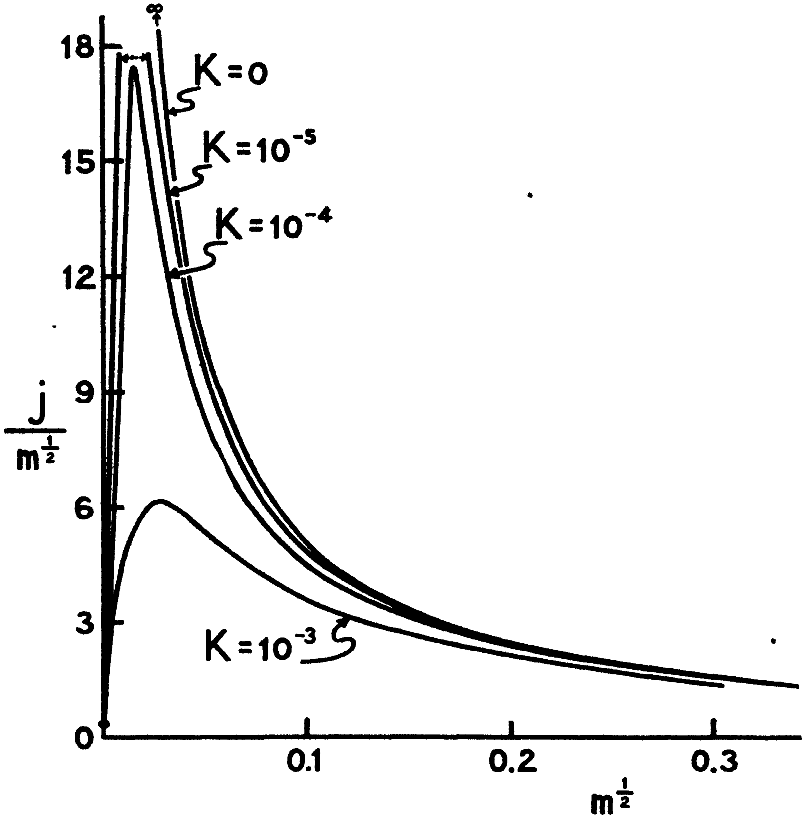

Fig. 7. Freezing point functions for electrolytes having various dissociation constants.

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[2] Since the completion of the calculations contained in this paper we have obtained values for the apparent dissociation constants of aspartic and glutamic acids at 0°. Use of the 25° values does not affect the values for

In Fig. 7 curves for $\frac{j}{m^{\frac{1}{2}}}$ against $m^{\frac{1}{2}}$ have been plotted with various values for $K$ assumed. The upper curve (designated $K = 0$) is for an acid having a zero dissociation constant and whose activity is proportional to the molality. In other words, this is the theoretical curve for a typical non-electrolyte which forms a perfect solution with water and whose activity is defined as though it dissociated into two ions. For such a perfect solution of a limiting weak electrolyte

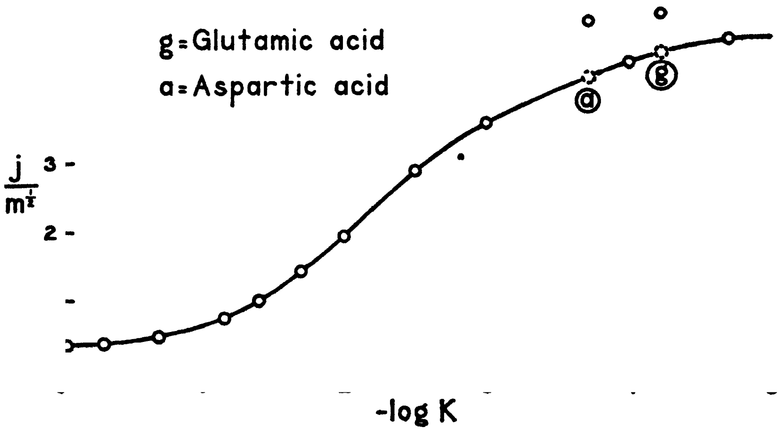

Fig. 8. A plot showing the relation between freezing point function at 0.01 M and the logarithm of the dissociation constant for weak electrolytes. The broken circles indicate theoretical values for aspartic and glutamic acid while the solid circles immediately above indicate the experimental values for the same substances. ○ = theoretical, ○ = actual.

$$\frac{j_2}{m^{\frac{1}{2}}} = \frac{0.5}{m^{\frac{1}{2}}} \tag{5}$$

since the value of $\nu$ is taken as equal to 2. In Fig. 3 of Randall and Allen's paper (14) it is to be noted that the values of $\frac{j_2}{m^{\frac{1}{2}}}$ at 0.01 M have been plotted only within the range in which their method of

the activity coefficients of the undissociated part which are given in this paper by more than about 3 per cent. C. L. A. S.

estimating $K$ from the freezing point data is most useful.[3] In Fig. 8 we have shown that if the plot is extended to substances having lower dissociation constants than $10^{-3}$ the curve becomes as asymptotic to $\frac{\dot{j}_2}{m^{\frac{1}{2}}} = 5$ (for $m = 0.01$).

The most striking result of these measurements is that the values of $\frac{\dot{j}_2}{m^{\frac{1}{2}}}$ for the two acids in concentrations greater than 0.01 M lie above the limiting curve, which in turn lies above the curve for acids having dissociation constants equal to that of either glutamic or aspartic acid. The change of dissociation constant between 25° and 0° is not larger than 2-fold and does not affect the relative position of the curves.

In the light of ordinary conventions and the values of $\frac{\theta}{m}$ given in Table II, or on the basis of our discussion of the values of $\frac{\dot{j}_2}{m^{\frac{1}{2}}}$, we see that these acids give in solution less than 1 molecule per aspartate or glutamate group, and this in molalities as low as 0.01 M. Neither the electrometric method nor the conductivity method of measuring the dissociation constant can of course tell us anything regarding the molecular weight of the undissociated acid. The assumption of ionic micelles alone, that is, the assumption that the aspartate ions formed by the dissociation of the acid agglomerate to form multicharged aggregates cannot explain a value of $\frac{\dot{j}_2}{m^{\frac{1}{2}}}$ less than 0.5, for this would still leave at least 1 molecule, namely the $H^+$ formed by the dissociation of each molecule of acid (3).

We shall now attempt to assign to the undissociated acids a degree of association (or of micellation), so imagined that the degree of association is smaller in the more dilute solution, and such that the correct measured freezing point lowering is obtained if the unassociated part and the associated part behave as perfect solutions and the dissociated part has the activity coefficient of other typical weak electrolytes. In the next section we shall see that there is some micelle formation in the solutions of

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[3] The results of this investigation show that the method of Randall and Allen (14) is applicable only to those weak electrolytes which are not aggregated in the unionized state.

the sodium salts, though the amount of micellation is small, and that at the ionic strength of the ions in the solutions of the pure weak acids this should be negligible. To this extent our assumption of no micelles in the dissociated part is justified. The assumption which we are making is similar to that of the neutral micelles of McBain and Salmon (17).

In Table II the interpolated freezing point lowerings of aspartic acid and of glutamic acid at three concentrations are given. By means of the dissociation constants and Equation 4 we have calculated these concentrations so that the ionized part is at a round molality and by interpolation of the data of Randall and Young (15) have determined the freezing point lowering of hydrochloric acid at these same ion concentrations. By subtracting this freezing point lowering due to the ions we determine the freezing point lowering due to the unionized part of the acids. We have here assumed that the freezing point lowering is the sum of independent effects due to the ions and the undissociated part, which assumption is justified by the calculations of Randall and Allen (14). We then substitute in the following equation[4]

$$j_u = 1 - \frac{\theta_u}{\nu_u \lambda m_u} \tag{6}$$

where $j_u$ is taken as zero to conform to the assumption of a perfect solution and solve for $\nu_u$. This value is given in Column 7 of Table III and represents the average number of undissociated groups per unionized aspartate or glutamate radical. In the case of the aspartic acid the value of $\nu_u$ is as we predicted for micelle formation, largest at the lowest concentration, showing dissociation of the neutral micelle. Over the concentration range used glutamic acid shows no decided change in the value for $\nu_u$ though the trend is the same as for aspartic acid.

Equation 6, in which the value for $j_u$ was placed equal to zero (Table III, Column 7), can be used in another manner. If $\nu_u$ be taken as unity ($\nu_u = 0$ when $m = 0$) a value of $j_u$ can be found and this divided by $m_u$ gives the freezing point function for the undis-

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[4] The calculation of $\nu_u$ by means of Equation 6 is only approximate. It involves the same errors as are made in calculating the degree of dissociation when the van't Hoff factor $i$ is used. See Lewis and Randall ((13) pp. 308, 341, and 346).

sociated part of the amino acids. This has been done in Columns 8 and 9 of Table III. These results are plotted in Fig. 9. Uncertainty in the total freezing point depressions of the acid solutions makes it impossible to give values at lower values of $m_u$. The activity coefficient of the undissociated part of the acids can be calculated with the aid of the equation

$$\log \gamma_u = -\frac{j_u}{2.303} - \frac{1}{2.303}\int_0^{m_u} \frac{j_u}{m_u}\, d\, m_u \tag{7}$$

TABLE III.

*Degree of Association and Freezing Point Function of the Non-Ionized Portion of Aspartic and Glutamic Acids.*

| Substance analysed. | $m_i$ (1) | $m_u$ (2) | $m$ ($m_i + m_u$) (3) | $\theta$ experimental (4) | $2\theta_i$ (5) | $\theta_u$ ($\theta$ experimental $-2\theta_i$) (6) | $\nu_u$[*] (7) | $j_u$[†] (8) | $\frac{j_u}{m_u}$ (9) |
|---|---|---|---|---|---|---|---|---|---|
| Aspartic acid. | 0.0016 | 0.01174 | 0.01334 | 0.02362 | 0.00586 | 0.01776 | 0.814 | 0.1855 | 15.80 |
| | 0.002 | 0.01817 | 0.02017 | 0.03420 | 0.00732 | 0.02688 | 0.798 | 0.2032 | 11.17 |
| | 0.0025 | 0.02810 | 0.03060 | 0.05020 | 0.00913 | 0.04110 | 0.787 | 0.2130 | 7.58 |
| Glutamic acid. | 0.000625 | 0.00616 | 0.00678 | 0.01232 | 0.00230 | 0.01002 | 0.877 | 0.1235 | 18.77 |
| | 0.0009 | 0.01263 | 0.01353 | 0.02404 | 0.00331 | 0.02073 | 0.883 | 0.1167 | 9.23 |
| | 0.0010 | 0.01555 | 0.01655 | 0.02910 | 0.00368 | 0.02543 | 0.881 | 0.1195 | 7.68 |
| | 0.00117 | 0.02133 | 0.0225 | 0.03913 | 0.00429 | 0.03484 | 0.880 | 0.1200 | 5.63 |

[*] $\nu_u$ is calculated from the equation $j_u = 1 - \frac{\theta_u}{\nu_u \lambda m_u} = 0$.

[†] $j_u$ is calculated from the equation $j_u = 1 - \frac{\theta_u}{\nu_u \lambda m_u}$ in which the value for $\nu_u$ is taken as unity.

In Table IV the calculations have been made at three round concentrations of the undissociated part of the acid solutions. Rather arbitrary extrapolations have been made in obtaining the areas under the curves of Fig. 9. The calculation of $\nu_u$ which we have made is based upon two assumptions concerning the dissociation constants (1) of the acids which cannot be entirely true. First, the value was calculated by assuming that the activity of the undissociated part of the acids was given by its molality. Later we shall show that the activity coefficient of the undissociated part of the acids is considerably less than unity due to the considerable degree of association. Second, the value for the dissociation constants determined for 25° was used for temperatures slightly below 0°. Since in other systems the degree of association is markedly dependent on temperature (2), an appreciable error must have resulted. The values in Table IV for $\gamma_u$ are probably accurate to within 10 per cent.

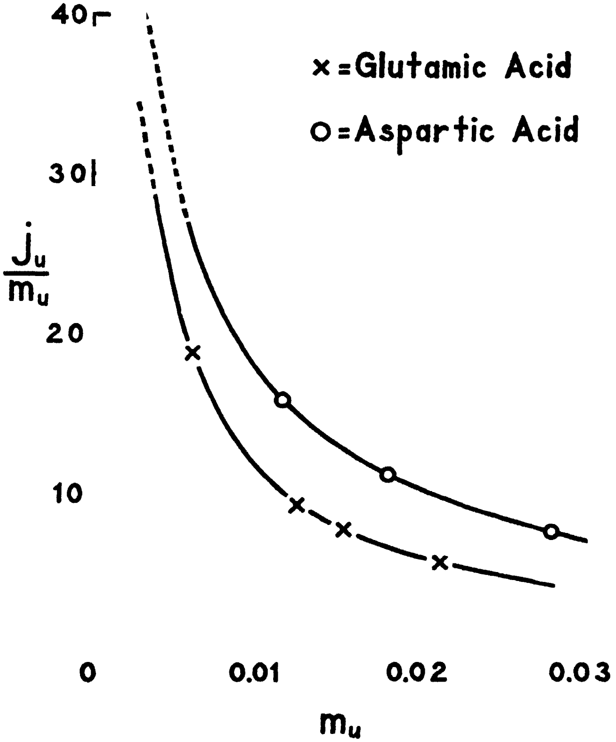

FIG. 9. Freezing point function of the undissociated part of aspartic and glutamic acids in solution.

*Significance of Dissociation Constants of an Acid Whose Unionized Part Forms Micelles.*—Any attempt to invent a mechanism (18) which will explain the thermodynamic behavior of a weak acid must of necessity become more arbitrary the more complicated the mechanism which it is necessary to postulate. The mere agreement of the results of the attempted mechanism with the experimental results, *i.e.* measured freezing point lowering, is not a proof of the actuality of the mechanism, for thermodynamics does not depend upon a mechanism. In the case of acetic acid it was found profitable to postulate undissociated and ionized parts of the solution and these are related through the dissociation constants and the activity coefficients of the ions in a very convenient manner. But just as it proved unprofitable to attempt to determine separately the concentration and activity coefficients of the parts of a strong electrolyte the number of empirical constants in the present example necessary to arrive at the measured freezing point is too large to be convenient. It would no doubt be possible by a method of approximations to so alter our dissociation constants and to make further assumptions so that the calculated result would more nearly give the measured freezing point at all concentrations. The curves $\frac{j_u}{m_u}$ of Fig. 9 would probably have much smaller values as $m \doteq 0$. Thus with our present values, $K\left(K = \frac{(m_{\pm}\ \gamma_{\pm})^2}{m_u\ \gamma_u}\right)$ is not constant at various concentrations. It was arbitrarily taken constant for $\gamma_u = 1$ in our original calculation of $m_u$.

TABLE IV.

*Activity Coefficients of the Undissociated Part of Aspartic and of Glutamic Acids.*

| Substance analyzed. | $m_u$ | $\frac{j_u}{m_u}$ | $j_u$ | $-\frac{j_u}{2.3}$ | $-\frac{1}{2.3}\int_0^{m_u} \frac{j_u}{m_u}\, d\, m_u$ | $\gamma_u$ |
|---|---|---|---|---|---|---|
| Aspartic acid. | 0.01 | 18.40 | 0.1840 | −0.0800 | −0.1378 | 0.606 |
| | 0.02 | 10.38 | 0.2076 | −0.0903 | −0.1971 | 0.516 |
| | 0.03 | 7.10 | 0.2130 | −0.0926 | −0.2348 | 0.471 |
| Glutamic acid. | 0.01 | 11.80 | 0.1180 | −0.0513 | −0.1022 | 0.702 |
| | 0.02 | 6.00 | 0.1200 | −0.0522 | −0.1391 | 0.644 |
| | 0.03 | 3.90 | 0.1170 | −0.0509 | −0.1604 | 0.615 |

We are thus forced to the expedient used for strong electrolytes; namely, to disregard the unionized part entirely and take $a_2$, the activity of the solute, equal to the product of the activities of the ions when these are defined in the ordinary manner. This procedure will give correct results independent of a mechanism. However, we may well continue to use our former conventions of an undissociated part for approximate calculations. Furthermore, in the present case these approximations will be less in error at room temperature than at 0° because the fraction micellated will be less. At the same time we must remember that the temperature coefficient of the "overall" activity coefficients (see $\gamma$, Table V) will be greater than that of strong electrolytes because the partial molal heat of dilution is greater.

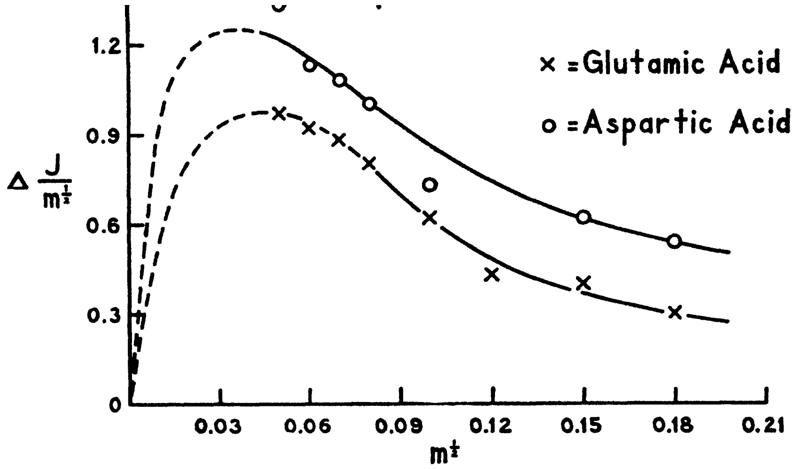

FIG. 10. A plot showing the differences in the areas between the theoretical and the experimental curves for the freezing point functions of aspartic and glutamic acids.

We now return to the main problem of extrapolating the curves of Figs. 5 and 6. We shall attempt to assign a reasonable value to the activity coefficient at 0.01 M which we believe to be accurate to within 10 per cent. We shall calculate the area to $m^{\frac{1}{2}} = 0.1$ M in two steps. The first is calculated as the area under the curve of an acid of $K_{\text{aspartic}} = 2 \times 10^{-4}$ and $K_{\text{glutamic}} = 6 \times 10^{-5}$. These areas are then increased by the area of the curve of Fig. 10 which represents the difference between the measured curve and the theoretical curve. This difference must become zero at infinite dilution where the micellation is nil. The error in our assumption probably leads to an error not greater than 5 per cent in the total area. Areas at concentrations lower than 0.01 M have been similarly obtained by proper subtraction of areas. By algebraically adding the quantities given in Columns 3, 4, and 5 of Table V the logarithm of the "overall" activity coefficient $\gamma$ is obtained. In Column 6 the actual values for $\gamma$ for the two amino acids are given. It is probable that the uncertainty of these values does not exceed 10 per cent at the lower concentrations and 5 per cent at the highest concentrations. In Column 7 we have given the interpolated values for the freezing point depression and in Column 8 values for the activity of the water ((13) p. 284) as calculated from the equation

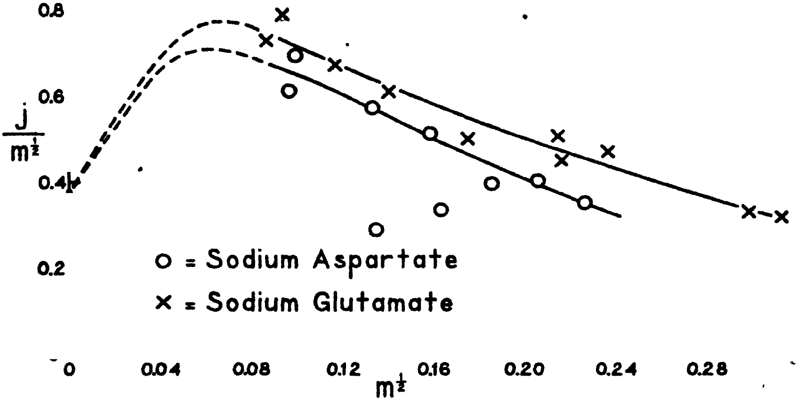

FIG. 11. Freezing point function for the monosodium salts of aspartic and glutamic acids.

$$a_1 = -0.004211\,\theta - 0.0000022\,\theta^2 \qquad (8)$$

*Activity Coefficients of the Monosodium Salts of Aspartic and of Glutamic Acid.*

In Fig. 11 the $\frac{j}{m^{\frac{1}{2}}}$ *versus* $m^{\frac{1}{2}}$ curves are shown for the monosodium salts of aspartic and glutamic acids. Somewhat arbitrary extrapolations to zero concentration as shown by the dotted portions of the curves have been made. This, however, affects the values of the activity coefficients to only a slight extent. The curves agree in trend with those for the sodium silicates (3) and the soaps (2)[5] and are interpreted as indicating the occurrence of association or micellation. That this association is ionic rather than molecular is indicated by the values of $\frac{\theta}{m}$ in Table II which are like those for strong electrolytes. However, there is no proof that molecular association does not occur also. The effect is quite small for the values of $\gamma$ for the two salts as given in Table V are but little below those for typical strong electrolytes. It does not seem feasible to attempt a calculation of the degree of association as was done for the free acids since we have no theoretical normal behavior on which to base the calculations. Any possible effect of this kind is included in our calculated "overall" activity coefficient. A comparison of $\gamma$ in Table V and $\frac{\Lambda}{\Lambda^{\circ}}$ in Table I with the corresponding values of a typical strong electrolyte shows that while the values of $\alpha$ for the monosodium salts of aspartic and of glutamic acid are about the same as that for a strong electrolyte, such as sodium chloride, the values of $\gamma$ are considerably lower, the effect probably being due to a small amount of micellation and to incomplete dissociation.

It is remarkable that the sodium salts of these relatively simple amino acids should show any noticeable degree of micellation. As shown by the curves of Fig. 11 sodium glutamate which contains 5 carbon atoms is slightly more micellated than sodium aspartate which contains 4 carbon atoms.

On the other hand, it is noted that the values of $\gamma$ for aspartic and for glutamic acid are much smaller than the values for $\alpha$. The value for $\frac{\gamma}{\alpha}$ is about 0.75 for aspartic and 0.55 for glutamic acid. The values of $K_{\Lambda} = \alpha^2 \frac{m}{(1 - \alpha)}$ given in the last column of

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[5] In the paper by Randall, McBain, and White (2) an error was made in calculating the values of $\frac{j}{m}$ at the lower concentrations and erroneous results for the activity coefficients were therefore obtained. Actually, the curves have the same shape as those of Fig. 11.

## TABLE V.

*Activity Coefficients of Aspartic and Glutamic Acids and Their Monosodium Salts at 0°.**

| $m$<br>(1) | $\frac{j}{m^{\frac{1}{2}}}$<br>(2) | $-\frac{j}{2.3}$<br>(3) | $-\frac{2}{2.3}\int_0^m$<br>(4) | $-\frac{2}{2.3}$ (Added area)<br>(5) | $\gamma$<br>(6) | $\theta$<br>(7) | $a_1$<br>(8) |
|---|---|---|---|---|---|---|---|
| **Aspartic acid.** | | | | | | | |
| 0.0025 | 8.93 | −0.1941 | −0.4549 | −0.0470 | 0.202 | 0.00515 | 0.99995 |
| 0.0050 | 6.89 | −0.2119 | −0.5755 | −0.0682 | 0.139 | 0.00952 | 0.99990 |
| 0.0075 | 5.76 | −0.2169 | −0.6541 | −0.0824 | 0.111 | 0.01396 | 0.99987 |
| 0.010 | 5.09 | −0.2214 | −0.7015 | −0.0913 | 0.097 | 0.01822 | 0.99982 |
| 0.015 | 4.34 | −0.2311 | −0.7797 | −0.1059 | 0.076 | 0.02612 | 0.99975 |
| 0.020 | 3.83 | −0.2355 | −0.8367 | −0.1163 | 0.065 | 0.03404 | 0.99966 |
| 0.025 | 3.50 | −0.2406 | −0.8807 | −0.1250 | 0.057 | 0.04149 | 0.99960 |
| 0.030 | 3.22 | −0.2425 | −0.9182 | −0.1326 | 0.051 | 0.04930 | 0.99952 |
| **Glutamic acid.** | | | | | | | |
| 0.0025 | 9.50 | −0.2063 | −0.5333 | −0.0334 | 0.169 | 0.00488 | 0.99995 |
| 0.0050 | 7.21 | −0.2215 | −0.666 | −0.0500 | 0.116 | 0.00911 | 0.99990 |
| 0.0075 | 6.04 | −0.2273 | −0.746 | −0.0609 | 0.092 | 0.01329 | 0.99988 |
| 0.01 | 5.21 | −0.2262 | −0.805 | −0.0687 | 0.080 | 0.01781 | 0.99982 |
| 0.015 | 4.28 | −0.2278 | −0.888 | −0.0794 | 0.064 | 0.02652 | 0.99975 |
| 0.020 | 3.75 | −0.2304 | −0.948 | −0.0851 | 0.055 | 0.03492 | 0.99965 |
| 0.025 | 3.40 | −0.2326 | −0.992 | −0.0903 | 0.048 | 0.04320 | 0.99957 |
| 0.030 | 3.10 | −0.2332 | −1.033 | −0.0945 | 0.044 | 0.05263 | 0.99949 |
| **Monosodium aspartate.** | | | | | | | |
| 0.01 | 0.625 | −0.0272 | −0.0539 | | 0.830 | 0.03484 | 0.99964 |
| 0.02 | 0.551 | −0.0339 | −0.0756 | | 0.777 | 0.06853 | 0.99933 |
| 0.03 | 0.469 | −0.0353 | −0.0903 | | 0.750 | 0.10243 | 0.99900 |
| 0.04 | 0.407 | −0.0354 | −0.1006 | | 0.731 | 0.13654 | 0.99868 |
| 0.05 | 0.357 | −0.0347 | −0.1079 | | 0.720 | 0.17097 | 0.99835 |
| 0.06 | 0.314 | −0.0334 | −0.1143 | | 0.711 | 0.20581 | 0.99800 |
| **Monosodium glutamate.** | | | | | | | |
| 0.01 | 0.715 | −0.0310 | −0.0568 | | 0.817 | 0.03601 | 0.99963 |
| 0.02 | 0.622 | −0.0382 | −0.0808 | | 0.760 | 0.07148 | 0.99930 |
| 0.03 | 0.554 | −0.0422 | −0.0970 | | 0.726 | 0.10681 | 0.99896 |
| 0.04 | 0.502 | −0.0437 | −0.1092 | | 0.703 | 0.14214 | 0.99862 |
| 0.05 | 0.460 | −0.0447 | −0.1191 | | 0.686 | 0.17750 | 0.99827 |
| 0.06 | 0.422 | −0.0450 | −0.1271 | | 0.673 | 0.21294 | 0.99797 |

* The activity coefficient $\gamma$ is calculated from the formula

$$\log \gamma = -\frac{j}{2.3} - \frac{2}{2.3}\int_0^m \frac{j_2}{m^{\frac{1}{2}}}\, d\, m^{\frac{1}{2}}.$$

Table I, show a tendency to a smaller value in the more concentrated solution. This tendency is also shown by acetic acid ((13) p. 310). The decrease in the case of the amino acids, however, is much more marked and occurs at far lower concentrations. Pure acetic acid is assumed to consist of aggregated $CH_3COOH$ groups, and it is only reasonable to assume that in fairly concentrated solutions the average degree of aggregation would be greater than unity. It is not unreasonable to suppose that pure, liquid supercooled aspartic and glutamic acids would consist of agglomerates which are much more stable and which probably contain a larger number of molecules than those of acetic acid. The reason that we are able to utilize with such great success the device of a dissociation constant in the case of acetic acid is because the agglomerates are practically completely dissociated into molecules containing a single $CH_3COOH$ group at concentrations as great as 0.1 molal.

Unless given otherwise the symbols used in this paper have the same meaning as given by Lewis and Randall ((13) p. 621).

## SUMMARY.

1. Conductivity data for solutions of aspartic and glutamic acids and their monosodium salts at 0° are given.

2. The transference numbers and the degree of ionization of aspartic and glutamic acids and their monosodium salts in solution at 0° have been calculated. The data indicate that the free acids are but little ionized while the sodium salts are highly ionized.

3. Freezing point determinations with solutions of aspartic and glutamic acids and their monosodium salts have been carried out.

4. These data have been used to calculate (*a*) the activity coefficients of aspartic and glutamic acids and their monosodium salts in solution, considering them as uniunivalent electrolytes; (*b*) the activity coefficients of the undissociated part of aspartic and glutamic acids in solution.

5. The data indicate that (*a*) the undissociated part of aspartic and glutamic acids in solutions exists to a considerable extent as neutral aggregates; (*b*) the ionized part of the monosodium salts of aspartic and glutamic acids in solution probably exists to a slight extent as ionic aggregates or micelles.

6. It is pointed out that the use of the "overall" activity coeffi-

cient avoids all the uncertainties involved in the arbitrary selection of a dissociation constant and gives a convenient measure of the activity of the solute.

7. It is further shown that with weak acids which form micelles the dissociation constant is not a convenient quantity for exact calculations.

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# STUDIES ON THE MODE OF COMBINATION OF IRON WITH CERTAIN PROTEINS, AMINO ACIDS, AND RELATED COMPOUNDS.*

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## INTRODUCTION.

The present work is a continuation of the studies which have been carried out in this laboratory on the mode of combination of proteins with the inorganic elements. Previous reports have dealt with the compounds of proteins with the alkali metals (1) and the alkaline earth elements (2). Other workers have carried out investigations on the combination of proteins or their derivatives with copper (3–18), silver (14, 19–25), zinc (24, 26–30), cobalt (16, 24, 31), cerium (32), lanthanum (18, 32), aluminum (16, 18), nickel (16), chromium (16), and gold (23). Our investigations have been extended to include the compounds which ferric iron forms with certain proteins, amino acids, and related substances.

Among the early investigators Bunge (33), Schmiedeberg (34), and Ascoli (35) report that iron forms complex compounds with proteins and certain related substances. The work of Weinland (36) and his associates is also indicative of the ability of iron to form complexes, especially with carboxylic acids. The investigations of Belloni (37), Uspenski and Uspenskaja (38), Hopkins and Wann (39), Reed and Rice (40), and Gile and Carrero (41) indicate that citrates and probably tartrates form complex ions with ferric iron. Dakin (11) showed that hydroxyaspartic acid will prevent the precipitation of iron in the presence of excess of alkali. A similar observation was made for glycerol by Fischer (42). In recent times Starkenstein (43) and Starkenstein and Weden (44) have sought to correlate the pharmacology of iron with the form in which it is combined. Starkenstein believes that only anodic iron is pharmacologically active. Certain of his results will be discussed later.

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Iron has been used as a precipitant for certain protein fractions by Müller (45) and by Röhmann and Shmamine (46). The work of Rona and Michaelis (47), Pauli and Flecker (48), Neumann (29), and Kondo and Hayashi (16) indicates that proteins will unite with iron to form undissociated salts. The ultrafiltration experiments of Heymann and Oppenheimer (23) and of Schorn (24) indicate that some interaction occurs between the albumin and ferric chloride. Bechhold (49) interprets this in terms of colloidal dispersion of ferric hydroxide. Freundlich and Lindau (50) interpret their experiments as indicating chemical combination between the protein and the iron.

While most of the evidence available favors the idea that proteins form compounds with ferric iron that are but slightly dissociated (which may be construed as indicating complex salt formation) the groupings in the protein molecule that are responsible for this union are not known. The work reported in this paper is an effort to determine the nature of the groups which may unite with ferric iron under certain definite conditions to form compounds having a low degree of dissociation. We have included a large number of compounds other than proteins and amino acids for the purpose of throwing light upon the factors involved in the formation of complex compounds of iron. The results are utilized in an attempt to account for the amount of iron with which certain proteins combine under definite conditions.

## EXPERIMENTAL.

Two procedures have been followed in the present investigation. The first one consists in determining the concentration of ferric ion in the presence of the substance being tested. The color produced with thiocyanate was utilized for this purpose. The second one consists in determining to which pole the iron migrates under the influence of an electric current.

All paH measurements have been made by means of the glass electrode. This electrode was chosen in preference to the hydrogen or the quinhydrone electrode in order to prevent any reducing effect on the ferric ions. The glass membranes were made in the manner described by MacInnes and Dole (51). One of the samples of soft glass that was in stock in this laboratory was found suitable. Its composition is not known. The electromotive force was measured by means of a vacuum tube potentiometer of the type described by Partridge (52). A few minor changes were made in his set-up in order to enable us to use a Leeds and Northrup student potentiometer instead of a voltmeter and a galvanometer instead of an ammeter. This arrangement proved very satisfactory. The results were accurate and reproducible to 1 millivolt or a little less than 0.02 paH unit.

The method employed in determining the ferric ion concentration consisted in adding the substances to be tested to a known solution of ferric iron. To this a standard amount of ammonium thiocyanate was added and the reaction was adjusted to a definite paH. The color of this solution was compared with that of a standard ferric thiocyanate solution. In every case the standard contained the same amount of thiocyanate and had the same paH as the solution compared with it. If the substance being tested formed a compound with iron (*e.g.* complex ion) which was less dissociated than ferric thiocyanate the color of the solution would be less intense than that of the standard. If the ferric compound was dissociated to a greater extent than ferric thiocyanate no appreciable effect on the color would be produced. It should be noted that at any one paH an equilibrium existed in these solutions between iron, thiocyanate, and the substance being tested. If any two of these and the paH were kept constant the effect of varying the concentration of the other could be determined.

Except in those cases where the effect of varying paH was studied, all solutions were adjusted to a paH of 2.5. This is about the upper limit at which this method can be used conveniently and accurately, due to the marked insolubility of ferric hydroxide and the susceptibility of the color to very slight changes in hydrogen ion concentration in more alkaline solutions.

In all, 61 substances have been tested. Thirty-five of these reduced the intensity of the color. The remainder either showed no effect or slightly increased the color. This increase is probably due to the change in solvent and is characteristic of those substances which possess a long carbon chain. Short carbon chains will produce the same effect at higher concentrations.

The results which were obtained with substances having an effect

TABLE I.

*Effect of Varying the Amount of Ammonium Thiocyanate and Acidity of the Solution.*

| Substance added. | Total volume. | Final paH. | Substance added. | 0.5 M $NH_4CSN$ added. | Iron present. | Iron determined. | Iron not free by difference. |
|---|---|---|---|---|---|---|---|
| | *cc.* | | *cc.* | *cc.* | *mg.* | *mg.* | *mg.* |
| 0.01 M lactic acid. | 25 | 2.5 | 1.0 | 1.0 | 1.12 | 0.81 | 0.31 |
| | 25 | 2.5 | 1.0 | 0.5 | 1.12 | 0.78 | 0.34 |
| | 25 | 2.5 | 1.0 | 2.0 | 1.12 | 0.90 | 0.22 |
| | 25 | 2.5 | 1.0 | 3.0 | 1.12 | 0.93 | 0.19 |
| | 25 | 2.5 | 1.0 | 4.0 | 1.12 | 0.94 | 0.18 |
| | 25 | 2.5 | 1.0 | 5.0 | 1.12 | 0.97 | 0.15 |
| | 25 | 1.0 | 2.0 | 1.0 | 1.12 | 1.12 | 0.00 |
| | 25 | 0.0 | 2.0 | 1.0 | 1.12 | 1.12 | 0.00 |
| 0.01 M β-hydroxybutyric acid. | 25 | 2.5 | 0.5 | 1.0 | 1.12 | 1.14 | −0.02 |
| | 25 | 2.5 | 1.0 | 1.0 | 1.12 | 1.15 | −0.03 |
| | 25 | 2.5 | 2.0 | 1.0 | 1.12 | 1.16 | −0.04 |
| | 25 | 2.5 | 5.0 | 1.0 | 1.12 | 1.14 | −0.02 |
| | 25 | 2.5 | 10.0 | 1.0 | 1.12 | 1.12 | 0.00 |
| | 25 | 2.5 | 15.0 | 1.0 | 1.12 | 1.12 | 0.00 |
| 0.01 M malonic acid. | 25 | 2.5 | 2.0 | 1.0 | 1.12 | 0.78 | 0.34 |
| | 25 | 2.5 | 2.0 | 5.0 | 1.12 | 0.81 | 0.31 |
| | 25 | 2.5 | 2.0 | 10.0 | 1.12 | 0.84 | 0.28 |
| | 25 | 1.0 | 2.0 | 1.0 | 1.12 | 1.12 | 0.00 |
| 0.005 M tartaric acid. | 25 | 2.5 | 0.5 | 0.5 | 1.12 | 0.87 | 0.25 |
| | 25 | 2.5 | 0.5 | 1.0 | 1.12 | 0.89 | 0.23 |
| | 25 | 2.5 | 0.5 | 2.0 | 1.12 | 0.90 | 0.22 |
| | 25 | 2.5 | 0.5 | 5.0 | 1.12 | 0.91 | 0.21 |
| | 25 | 2.5 | 0.5 | 10.0 | 1.12 | 0.97 | 0.15 |
| | 25 | 1.0 | 0.5 | 1.0 | 1.12 | 1.12 | 0.00 |
| 0.0025 M glycerophosphoric acid. | 25 | 2.5 | 2.0 | 1.0 | 1.12 | 0.80 | 0.32 |
| | 25 | 2.5 | 2.0 | 5.0 | 1.12 | 0.86 | 0.26 |
| | 25 | 2.5 | 2.0 | 10.0 | 1.12 | 0.95 | 0.17 |
| | 25 | 1.0 | 2.0 | 1.0 | 1.12 | 1.12 | 0.00 |
| 0.01 M aspartic acid. | 25 | 2.5 | 2.0 | 0.5 | 1.12 | 0.89 | 0.23 |
| | 25 | 2.5 | 2.0 | 1.0 | 1.12 | 0.92 | 0.20 |
| | 25 | 2.5 | 2.0 | 2.0 | 1.12 | 1.01 | 0.11 |
| | 25 | 2.5 | 2.0 | 5.0 | 1.12 | 1.07 | 0.05 |
| | 25 | 1.0 | 2.0 | 1.0 | 1.12 | 1.12 | 0.00 |

TABLE I—*Concluded.*

| Substance added. | Total volume. | Final paH. | Substance added. | 0.5 M $NH_4SCN$ added. | Iron present. | Iron determined. | Iron not free by difference. |
|---|---|---|---|---|---|---|---|
| | *cc.* | | *cc.* | *cc.* | *mg.* | *mg.* | *mg.* |
| 1.52 per cent gelatin* (dry weight). | 25 | 2.5 | 2.0 | 1.0 | 1.12 | 0.79 | 0.33 |
| | 25 | 2.5 | 2.0 | 3.0 | 1.12 | 0.86 | 0.26 |
| | 25 | 1.0 | 2.0 | 1.0 | 1.12 | 1.12 | 0.00 |
| 1 per cent protamine. | 25 | 2.5 | 5.0 | 1.0 | 1.12 | 0.95 | 0.17 |
| | 25 | 2.5 | 5.0 | 5.0 | 1.12 | 1.08 | 0.04 |
| | 25 | 2.5 | 5.0 | 10.0 | 1.12 | 1.12 | 0.00 |
| | 25 | 1.0 | 5.0 | 1.0 | 1.12 | 1.02 | 0.10 |
| | 25 | 0.0 | 5.0 | 1.0 | 1.12 | 1.02 | 0.10 |

* The $NH_4SCN$ was 0.25 M in this case.

in decreasing the color[1] of the ferric thiocyanate solution are presented graphically in Figs. 1 to 7. It should be noted that in these experiments the amount of ammonium thiocyanate was kept constant. The effect of varying the amount of ammonium thiocyanate and the acidity of the solution on the color of the mixture of iron thiocyanate and the individual substances tested is shown by the data given in Table I. In order not to make the table unduly lengthy the data for only one substance from each group of substances tested have been included.

It is apparent from Fig. 1 that those substances which possess a hydroxyl group alpha to a carboxyl group markedly reduce the color of the ferric thiocyanate solution. Lactic acid is markedly effective in this respect. The corresponding unsubstituted acid, propionic, shows no effect on the color. This is not due to the fact that lactic acid is more highly ionized at the acidity employed since no effect in producing a color change was shown by chloroacetic acid which is a stronger acid than lactic acid. The data in Table I show that on increasing the amount of ammonium thiocyanate in the mixture of ferric thiocyanate and lactic acid

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[1] In the text we have used the expression "influencing the color" or "the effect on the color of the ferric thiocyanate" while in the figures the term "iron bound" is used. The amount of color change produced by a substance under test was taken as a measure of the amount of iron bound in the form of a compound which is less dissociated than ferric thiocyanate.

the effect of the lactic acid in reducing the color is decreased. This is probably due to a shift in the equilibrium towards ferric thiocyanate. Increasing the acidity of the solution decreases the effect of the lactic acid due probably to the formation of un-ionized lactic acid from the iron combination.

Fig. 1 also shows that if the hydroxyl group be moved back to the β position with respect to the carboxyl group as in hydracrylic acid the effect is much less pronounced. An analogous situation is found with the butyric acids. Butyric acid shows no effect. The effect produced by α-hydroxybutyric acid is almost as marked as lactic acid. On the other hand addition of β-hydroxybutyric acid[2] leads at first to a darkening of the color (see Table I) but the fact that this effect is overcome as the concentration of the acid

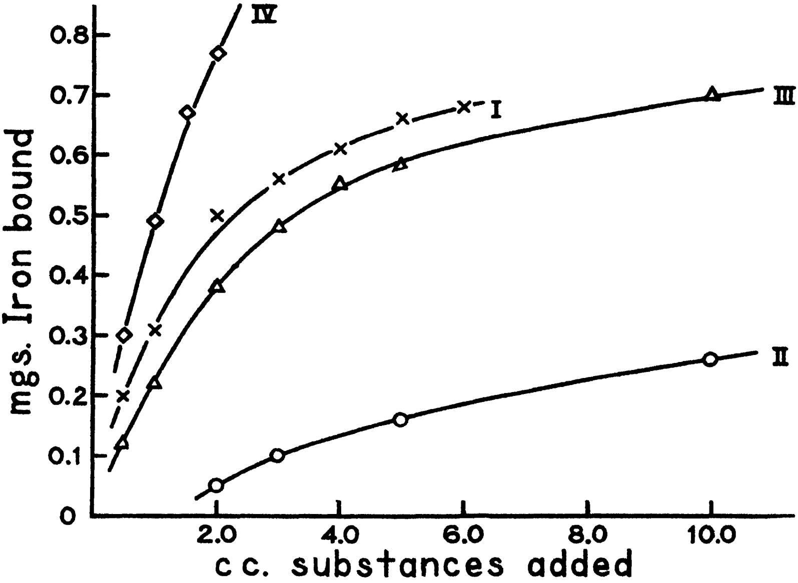

Fig. 1. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 0.01 M lactic acid; Curve II, 0.01 M hydracrylic acid; Curve III, 0.01 M α-hydroxybutyric acid; Curve IV, 0.01 M mandelic acid.

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[2] Kindly supplied to us by Dr. P. A. Levene.

is increased indicates that it also exhibits a tendency to decrease the color of the ferric thiocyanate solution. Another α-hydroxy acid, mandelic, exerts a marked effect in decreasing the color of the solution. The corresponding unsubstituted acids, phenylacetic and *p*-hydroxyphenylacetic, show no effect.

The effect of the dicarboxylic acids is indicated in Fig. 2. It is evident that their structure is similar to that of the hydroxy acids. Thus in oxalic acid

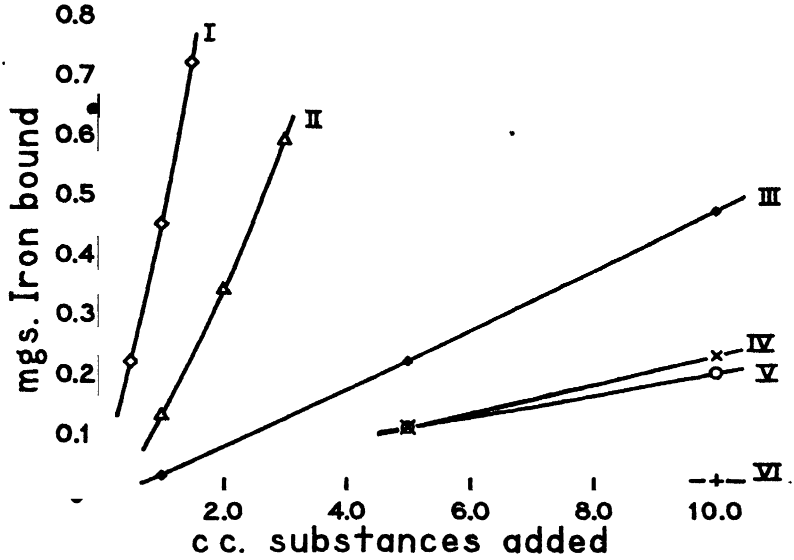
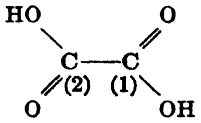


Fig. 2. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 0.005 M oxalic acid; Curve II, 0.01 M malonic acid; Curve III, 0.01 M maleic acid; Curve IV, 0.01 M succinic acid; Curve V, 0.01 M glutaric acid; Curve VI, 0.01 M adipic acid.



the hydroxyl group on carbon (2) is alpha to carboxyl group (1) and similarly the hydroxyl group on carbon (1) is alpha to carboxyl group (2). From this it is to be expected that oxalic acid would show a pronounced effect in decreasing the color of the ferric thiocyanate mixture. The curve bears out the prediction. It may be argued that oxalic acid decreases the color by reducing the ferric iron to the ferrous condition. However, the fact that the color can be restored after some minutes by the addition of hydrochloric acid to a paH of 0.0 argues against this. If the oxalic acid is added to the iron solution and the mixture is heated in boiling water for 15 minutes and then subjected to the thiocyanate test the color cannot be produced by the addition of hydrochloric acid to paH 0.0. This indicates that while reduction takes place at 100° it occurs very slowly, if at all, at room temperature. The fact that various investigators (36, 53, 54) have obtained complex salts of ferric iron with oxalates lends further support to the idea that we are dealing here with such a complex.

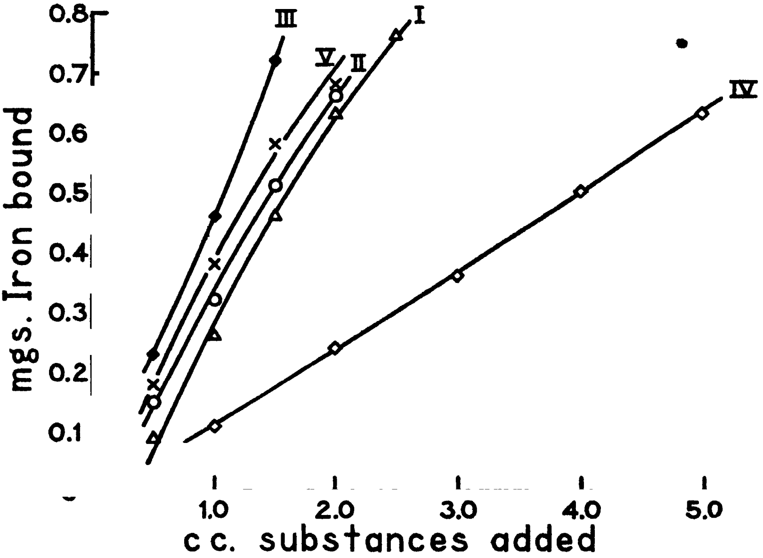


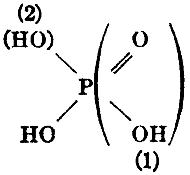
FIG. 3. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 0.0025 M citric acid; Curve II, 0.005 M malic acid; Curve III, 0.005 M tartaric acid; Curve IV, 0.0025 M gluconic acid; Curve V, 0.0025 M saccharic acid.

From the curves shown in Fig. 2 it is evident that the introduction of $CH_2$ groups into the dicarboxylic acid molecule leads to a decrease in the ability to affect the color of the ferric thiocyanate solution. Thus malonic acid is less potent than oxalic acid. Complex ferrimalonates are well known (36, 55). It is to be noted that maleic acid shows a greater effect than the saturated succinic acid. An explanation for this will be advanced later. The data for malonic acid in Table I indicate that the effect of increased amounts of ammonium thiocyanate or of hydrogen ions is to decrease the effect of malonic acid.

The curves represented in Fig. 3 show that if a hydroxyl group is present in addition to more than one carboxyl group the effect which is shown is very great. Attention is called to the fact that the molality of the solutions used and the abscissa scale employed differ from those for the preceding substances. The effect of an additional hydroxyl group is well illustrated by a comparison of the results for malic acid with those for tartaric acid. As in other instances the addition of more ammonium thiocyanate or of hydrogen ions decreases the effect of a given quantity of tartaric acid (see Table I).

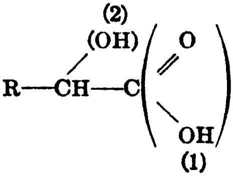
Substances which possess hydroxyl groups, but which either are not acids or are very weak acids, such as glucose, glycerol, dihydroxyacetone, and boric acid exert no effect on the color. These results do not signify that the substances in question do not combine with ferric iron. They indicate that under the conditions of our experiments these substances are not effective in reducing the color of the ferric thiocyanate solution. Levulose was found to exert a slight effect.

Certain inorganic acids, such as orthophosphoric, have a structure very similar to hydroxy organic acids. Their structures may be represented by the following formulæ.



$$\begin{matrix}\overset{(2)}{(\mathrm{HO})}\\ \mathrm{HO}\end{matrix}\!>\!\mathrm{P}\left(\begin{matrix}=\mathrm{O}\\ -\underset{(1)}{\mathrm{OH}}\end{matrix}\right)$$

and



$$\mathrm{R{-}}\overset{\overset{(2)}{(\mathrm{OH})}}{\mathrm{CH}}\mathrm{{-}C}\left(\begin{matrix}=\mathrm{O}\\ -\underset{(1)}{\mathrm{OH}}\end{matrix}\right)$$

The groupings numbered alike are analogous. In organic acids the hydroxyl group is in the *α* position with respect to the carboxyl;

in phosphoric acid it is on the same atom. In addition, phosphoric acid possesses another hydroxyl group. It was shown that a shift of the hydroxyl group from the α to the β position led to a decreased ability to affect the color of the ferric thiocyanate solution. Conversely, it may be expected that the effectiveness is increased when the hydroxyl groups are attached to the phosphorus atom as in phosphoric acid. The curves shown in Fig. 4

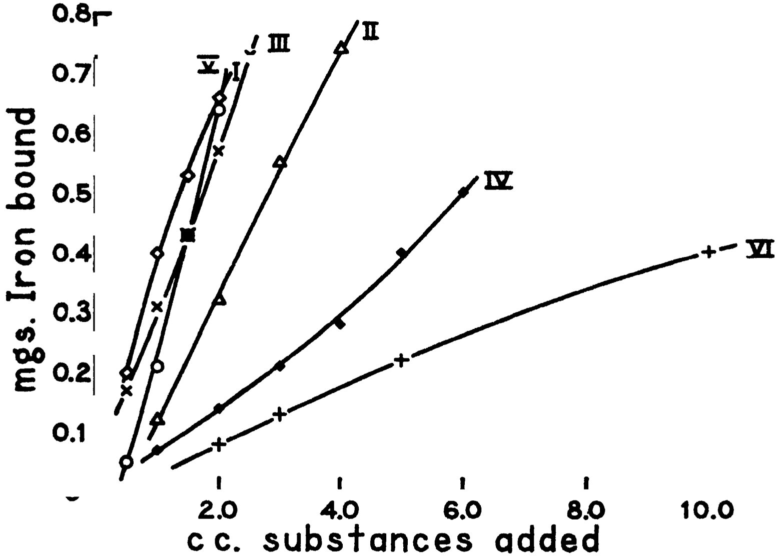


Fig. 4. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 0.0025 M orthophosphoric acid; Curve II, 0.0025 M glycerophosphoric acid; Curve III, 0.0025 M pyrophosphoric acid; Curve IV, 0.0025 M metaphosphoric acid; Curve V, 0.005 M arsenic acid; Curve VI, 0.01 M sulfuric acid.

bear out these statements. Complex iron phosphates are well known (36, 56).

If one of the hydroxyl groups of orthophosphoric acid is replaced by glycerol the resulting glycerophosphoric acid shows a lessened effect on the color of the ferric thiocyanate solution. In pyrophosphoric acid 2 molecules of orthophosphoric acid are united by the splitting off of a hydroxyl group from each. It is to be expected that its potency should be twice as great as that of glycerophosphoric acid. The data represented in Fig. 4 show that this is the order of magnitude. Metaphosphoric acid is less effective. The structure of arsenic acid is analogous to orthophosphoric acid. Its behavior also is similar to that of orthophosphoric acid. Sulfuric acid shows a slight effect on ferric thiocyanate. The result of increasing the concentration of ammonium thiocyanate or of hydrogen ions on the effect of a constant amount of glycerophosphoric acid (see Table I) is the same as in previously cited instances.

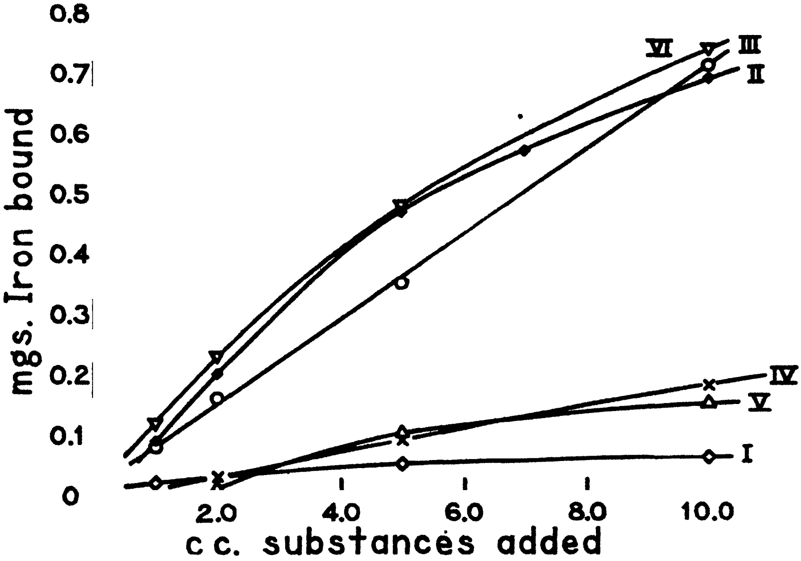


Fig. 5. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 0.01 M serine; Curve II, 0.01 M aspartic acid; Curve III, 0.01 M glutamic acid; Curve IV, 0.01 M asparagine; Curve V, 0.01 M creatine; Curve VI, 0.01 M β-hydroxyglutamic acid.

The addition of glycine, alanine, δ-amino-valeric acid, tyrosine, oxyproline, lysine, arginine, histidine, and tryptophane respectively did not lead to a decrease in the color of the ferric thiocyanate solution. In each instance a 0.01 molar solution of the amino acid was employed and as much as 10 cc. were added to make a total volume of 25 cc. Glycine anhydride, glycylglycine, and leucylglycine showed no decreasing effect on the color of the ferric thiocyanate solution. The effect of cystine was not determined on account of its insolubility at this acidity. Cysteine was not used because of the oxidation-reduction system which is set up in such solutions. It is known, however, that these two sulfur-containing amino acids do unite with iron (7, 8, 57–60). Taurine, cysteic acid, and isethionic acid all produce a slight darkening of the red color of the ferric thiocyanate solution.

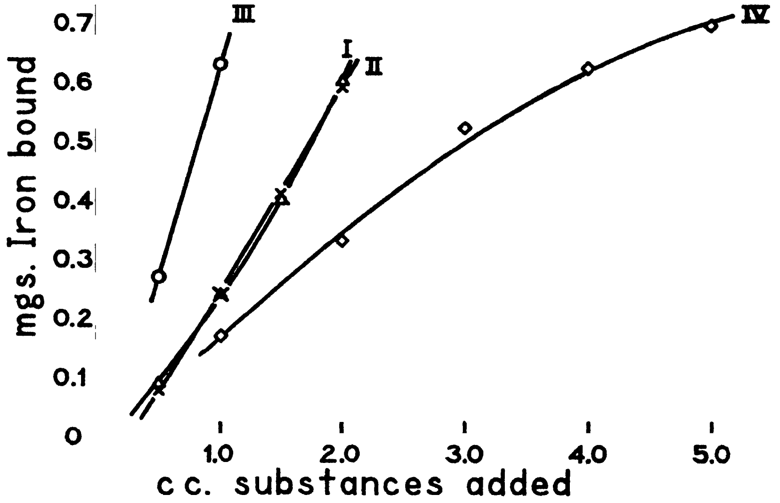


Fig. 6. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 0.94 per cent casein; Curve II, 1.00 per cent dephosphorized casein; Curve III, 1.00 per cent dephosphorized casein plus the phosphoric acid split off from it; Curve IV, 1.52 per cent gelatin.

Fig. 5 shows that the effect of the addition of serine is slight but definite. The dicarboxylic acids, aspartic, glutamic, and hydroxyglutamic, show a marked effect. It should be noted that this effect is much greater than that of the corresponding unsubstituted dicarboxylic acids. Asparagine shows a smaller effect than the corresponding amino acid. It might be expected from the similarity of the guanido group $\left(-\mathrm{C}\begin{matrix}/\!\!/\mathrm{NH}\\ \diagdown\mathrm{NH_2}\end{matrix}\right)$ of arginine to a carboxyl group that this amino acid should exhibit some effect. However, none was detected with a 0.01 molar solution. Creatine possesses this same group but it is much closer to the carboxyl group. It exhibits a slight effect.

Casein dissolved in a 5.0 molar urea solution to a concentration of 1 per cent produces the marked effect shown by Curve I in Fig. 6. Similar amounts of the urea solution alone produce only a slight darkening. The casein employed was prepared from milk by the Van Slyke and Baker (61) procedure with the modifications introduced by Greenberg and Schmidt (1). Its ash content was 0.21 per cent. The effect of casein dephosphorized by treating it with alkali as outlined by Rimington (62) was not materially different from casein. The original casein contained 0.81 per cent of phosphorus when estimated by the Fiske and Subbarow method. The phosphorus content of dephosphorized casein was too small to estimate. It might be concluded that the phosphorus as it occurs

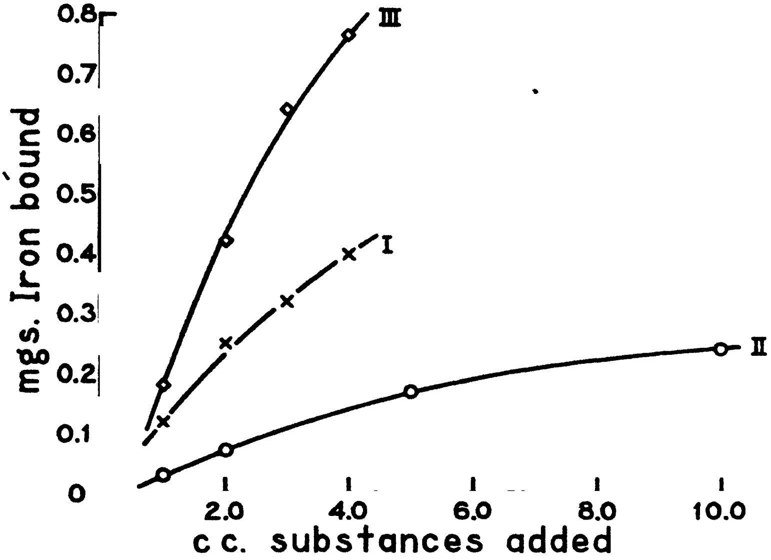


Fig. 7. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 1 per cent protamine sulfate; Curve II, 1 per cent protamine; Curve III, 0.0025 M yeast nucleic acid.

in casein does not participate in reducing the color of the ferric thiocyanate solution. However, as will be pointed out later this is probably not the case.

The effect of a gelatin solution was much less than the effect of the solution of casein. The gelatin contained 0.05 per cent of ash and was free from phosphorus. It was prepared according to the procedure described by Northrop and Kunitz (63).

The data relating to protamine and yeast nucleic acid are shown graphically in Fig. 7. It will be noted that a not inconsiderable part of the effect produced by protamine sulfate on the color of ferric thiocyanate is due to the presence of the sulfate radical. Removal of sulfuric acid by addition of barium led to a decreased effect. Yeast nucleic acid is very sparingly soluble in aqueous solution at the standard acidity employed but it dissolves readily in a 5.0 molar urea solution. When ferric chloride is added a precipitate containing iron forms. The results shown in Fig. 7 were obtained by centrifuging to remove the precipitate after addition of ammonium thiocyanate. The supernatant liquid was used for the color comparison.

The migration experiments were designed to check the results which were obtained from the experiments with ammonium thiocyanate. If the iron in a solution containing (in addition to the iron) only the substance under test, is present in the anodic condition as shown by migration experiments it must be combined in the form of a complex ion. On account of the acidity of the solutions employed it was not found feasible to carry out quantitative transference experiments.

In those cases where a sufficiently acid substance was being employed the procedure used was as follows: The substance was placed in contact with an excess of freshly precipitated ferric hydroxide and permitted to stand for several hours. The excess ferric hydroxide was then removed by centrifuging. The clear solution was then placed in a three compartment transference cell of the type described by Greenberg and Schmidt (1) and a current of several millivolts was passed for a period of several hours. Platinum gauze was used as electrodes. The solution was then separated into three portions and each was analyzed for iron. The sum of the iron contained in the three compartments should equal that contained in the original solution. If the substance to be tested was not sufficiently acid to react with ferric hydroxide (*e.g.* casein) then ferric chloride was used instead. It was necessary to add less ferric chloride than would react with the casein in order that no excess of ionic iron was present. When it was desired to carry out migration experiments at a lower acidity alkali was added to the solution and any ferric hydroxide which was formed was removed. The presence of other electrolytes did not lead to any difficulty since transference experiments were not attempted.

The iron was estimated colorimetrically as the thiocyanate. In some cases this could be done by the addition of a sufficient excess of hydrochloric acid to overcome the effect of the substance under test. In other cases it was necessary to ash the residue from the solution or to oxidize the material directly with potassium permanganate, the iron serving as a catalyst for the oxidation. In the case of pyrophosphate it was necessary to hydrolyze with strong hydrochloric acid. Care had to be taken, particularly in the cathode compartment, to make certain that all of the iron was in the ferric condition.

The results are presented in Table II. It will be seen that the iron dissolved in an acetic acid solution is cathodic at paH 2.5. If the acidity of the solution is decreased ferric hydroxide is precipitated. The experiments with lactic acid show that at paH 2.0 the iron is cathodic but if the acidity of the solution is increased to paH 7.1 the iron becomes anodic. A decrease in the acidity of the solution to paH 8.4 leads to precipitation of the iron as the hydroxide. The data for mandelic acid are similar to those obtained with lactic acid.

In the case of the dicarboxylic acids, oxalic and malonic, the iron is anodic even at a paH as low as 2.0. Malonic acid is unable to prevent the precipitation of ferric hydroxide at paH 6.0, but oxalic acid retains some iron at paH 6.5. As indicated in Table II the iron is also anodic at this acidity.

The iron in a citric acid solution is anodic at paH values of 2.1 and 7.0. It precipitates as the hydroxide at paH 8.1. Tartaric acid also holds the iron as an anion at paH values of 2.1, 7.0, and 10.0. It is exceedingly difficult to precipitate this iron as the hydroxide. Experiments showed that with the concentrations given in Table II under paH 10.0 it was necessary to make the solution 3.0 molar with sodium hydroxide before the iron precipitated. If saccharic

TABLE II.

*Migration of Iron in Solutions Containing Various Substances.*

| Substance added. | Original solution. | | | | Anode portion. | | | Middle portion. | | | Cathode portion. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | paH | Molality of substance being tested. | Iron per cc. | Total iron. | paH | Iron per cc. | Total iron. | paH | Iron per cc. | Total iron. | paH | Iron per cc. | Total iron. |
| | | | *mg.* | *mg.* | | *mg.* | *mg.* | | *mg.* | *mg.* | | *mg.* | *mg.* |
| Acetic acid. | 2.5 | 1.0 | 1.97 | 246.00 | 2.4 | 1.77 | 72.50 | 2.4 | 1.97 | 80.77 | 2.8 | 2.24 | 96.20 |
| Lactic acid. | 2.0 | 0.1 | 1.04 | 130.00 | 1.8 | 0.79 | 33.97 | 2.0 | 1.04 | 43.68 | 2.5 | 1.29 | 51.60 |
| | 7.1 | 0.1 | 0.54 | 62.91 | 4.0 | 0.57 | 21.09 | 6.0 | 0.50 | 21.25 | 10.7 | 0.50 | 18.50 |
| Mandelic acid. | 1.9 | 0.1 | 0.49 | 60.27 | 1.7 | 0.46 | 19.32 | 2.3 | 0.41 | 16.40 | 2.0 | 0.55 | 22.55 |
| | 7.0 | 0.1 | 0.24 | 27.24 | 3.7 | 0.25 | 9.00 | 9.0 | 0.21 | 8.51 | 11.4 | 0.22 | 8.14 |
| Oxalic acid. | 2.1 | 0.01 | 0.49 | 60.76 | 1.6 | 0.63 | 26.15 | 1.9 | 0.51 | 21.42 | 4.5 | 0.39 | 15.80 |
| | 6.5 | 0.1 | 0.10 | 11.20 | 2.7 | 0.12 | 4.32 | 9.0 | 0.08 | 3.20 | 10.0 | 0.09 | 3.24 |
| Malonic acid. | 1.5 | 0.1 | 1.45 | 175.45 | 1.4 | 1.60 | 64.00 | 1.6 | 1.51 | 60.40 | 1.8 | 1.18 | 48.38 |
| Citric acid. | 2.1 | 0.01 | 0.31 | 37.98 | 1.9 | 0.46 | 19.32 | 3.1 | 0.33 | 13.04 | 4.9 | 0.16 | 6.56 |
| | 7.0 | 0.1 | 0.51 | 57.63 | 4.0 | 0.55 | 19.80 | 9.0 | 0.49 | 20.09 | 9.6 | 0.44 | 15.84 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tartaric acid. | 2 1 | 0.01 | 0.25 | 30.25 | 2 0 | 0 27 | 10 53 | 2 0 | 0 28 | 11.48 | 2 8 | 0.22 | 9.02 |
| | 7.0 | 0 1 | 0.61 | 68 32 | 2 5 | 0 76 | 27.36 | 7.0 | 0 55 | 22 00 | 9 5 | 0.46 | 16.56 |
| | 10 0 | 0 1 | 0 58 | 63 51 | 3 5 | 0 66 | 23.10 | 10.3 | 0.54 | 21 33 | 10.4 | 0.51 | 17.85 |
| Saccharic acid. | 10 7 | 0 1 | 0 20 | 21 20 | 4 1 | 0.22 | 7 26 | 10.7 | 0.19 | 7.60 | 10.8 | 0.17 | 5.61 |
| Orthophosphoric acid. | 1 9 | 0 1 | 0 25 | 32 25 | 1 9 | 0.18 | 7.56 | 1.9 | 0.24 | 9.84 | 2.0 | 0.28 | 12.88 |
| Pyrophosphoric acid. | 1 4 | 0.1 | 0.14 | 17.36 | 1 0 | 0.20 | 8 40 | 1.4 | 0.14 | 5.74 | 1.9 | 0.09 | 3.69 |
| Glycerophosphoric acid. | 1.5 | 0.1 | 1 03 | 128 24 | 1.5 | 0.94 | 39.48 | 1.4 | 0.97 | 39.29 | 1.7 | 1.13 | 47.46 |
| | | | 3.50* | 435 75* | | 3.90* | 163 80* | | 3.50* | 141.75* | | 3.30* | 138.60* |
| | 3.7 | 0 1 | 0 56 | 70.00 | 3.4 | 0.59 | 24 49 | 5.0 | 0.56 | 23.52 | 6.3 | 0.51 | 21.17 |
| | 6 5 | 0.1 | 0.75 | 69 75 | 5 4 | 0.80 | 26.40 | 6.7 | 0.72 | 19 08 | 10.5 | 0.69 | 23.12 |
| Glutamic acid. | 2.7 | 0.075 | 0 15 | 18.30 | 2 4 | 0.06 | 2.52 | 3.1 | 0.12 | 4.68 | 3.4 | 0.24 | 9.84 |
| | 4 0 | 0.075 | 0.15 | 18 68 | 2 6 | 0.10 | 4 25 | 4.2 | 0.16 | 6.40 | 7.5 | 0.19 | 7.98 |
| | 5.0 | | + | | 3.0 | − | | 5.2 | + | | 7.8 | ++ | |
| Aspartic acid. | 7.0 | | + | | | ++ | | • | + | | | − | |
| | 7.4 | | + | | | ++ | | | + | | | − | |
| Casein. | 2.4 | 1% | 0 26 | 28.73 | 2.3 | 0.20 | 7.00 | 2.6 | 0.24 | 9.96 | 3.8 | 0.30 | 10.20 |
| | 7.2 | 1% | 0.17 | 15.73 | 5.0 | 0.20 | 6.60 | 8.3 | 0.18 | 5.04 | 10.0 | 0.14 | 4.41 |
| Gelatin. | 2.2 | 1% | 0.18 | 20 34 | 1.7 | 0.16 | 5.76 | 4.1 | 0:20 | 8.20 | 2.5 | 0.22 | 7.94 |
| | 7.0 | 1% | 0 19 | 20.90 | 3.8 | 0.20 | 6.80 | 7.7 | 0.20 | 8.40 | 7.5 | 0.18 | 6.12 |

* These figures refer to estimations of phosphorus.

acid is employed it is still more difficult to precipitate the iron. Indeed, in the concentrations shown in Table II it was found impossible to precipitate ferric hydroxide even by making the solution 10 molar with respect to sodium hydroxide. At acid reactions these solutions of ferric iron in tartaric and saccharic acids are yellowish and greenish yellow respectively. In alkaline solutions beginning about paH 10.0 for tartaric acid and higher for saccharic these solutions become a brilliant red. The tartaric solution remains red until the iron precipitates, but the saccharic acid loses its red color at high alkalinities and again assumes the greenish yellow color.

It may be observed from Table II that the iron in a solution of orthophosphoric acid is cathodic at a paH of 1.9. Similarly, the iron in a glycerophosphoric acid solution is cathodic at paH 1.5, but the iron in a pyrophosphoric acid solution is anodic at this same acidity. Phosphorus determinations on the glycerophosphoric acid solution at this paH show that it is anodic. If the paH of the glycerophosphoric acid solution is increased to 3.7 the iron becomes anodic and remains so at paH 7.0. The orthophosphoric acid solution cannot be similarly treated because ferric phosphate precipitates at paH 3.0.

A 0.1 molar solution of glycine, alanine, or arginine will not prevent the precipitation of ferric hydroxide at paH 5.0. Glutamic acid will retain some iron in solution at paH 5.0, but it is all precipitated at paH 6.0. As Table II shows this iron is cathodic at paH values of 2.7, 4.0, and 5.0. Aspartic acid retains a slight amount of iron even at paH 7.4. The amount is too small to determine accurately, but it seems to be definitely anodic.

The iron in solutions of casein or gelatin moves with the protein, the direction depending on the paH of the solution.

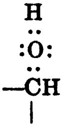
An explanation of these results will be found in the following section.

## DISCUSSION.

In order that a ferric compound may be undissociated in the presence of an excess of thiocyanate it is necessary that some force, in addition to the usual valence bond, be acting upon the iron atom to hold it in position. We may call this force secondary valence, coordinative valence, the attraction of charges, or what you will.

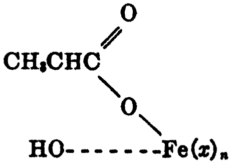
If it is not present the iron will dissociate and form ferric thiocyanate. We saw that adding propionic acid to a solution of an iron salt had no effect upon the subsequent color produced with ammonium thiocyanate. Similar amounts of lactic acid, however, markedly reduced the color. We must ascribe this difference in behavior to the hydroxyl group of the lactic acid. How does it produce this effect?

We know that oxygen can become tetravalent and it may do so here. A simpler explanation, however, and one more in accord with our data would seem to be the following. It is possible to compute our residual charge on this oxygen atom in the manner proposed by Latimer and Porter (64). We may briefly illustrate their method. The electronic arrangement of this atom is as follows:

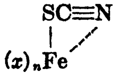


$$\begin{array}{c} \mathrm{H} \\ :\ddot{\mathrm{O}}: \\ -\ddot{\mathrm{C}}\mathrm{H} \\ | \end{array}$$

The oxygen atom has a kernel charge of 6+. We can see that it has 4 electrons free, 2 shared with hydrogen and 2 shared with carbon. The hydrogen ion is considered sufficiently close to the center of the negative charges between it and the oxygen to just neutralize 1 electron. The 2 electrons shared with carbon will be shared not equally, but in the ratio of the kernel charges of oxygen and carbon. These are 6+ and 4+ respectively so that the oxygen will have 0.6 of these 2 electrons. It we sum up our positive and negative charges we have, $6 - 4 - (2 \times \frac{1}{2}) - (2 \times 0.6) = -0.2$. According to this calculation the oxygen atom actually possesses a residual negative charge. This will exert an attraction on the positive iron atom and we may represent the compound formed as follows:



The $x$ here represents any negative group. This attraction in addition to the usual bond may be sufficient to prevent the dissociation of iron. If we consider that the thiocyanide ion has the structure $(SC \equiv N)^-$ then ferric thiocyanate itself may be a compound of the type above. Its structure would then be

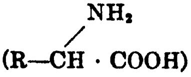


If the hydroxyl group in a hydroxy acid is moved back to the $\beta$-carbon atom, its residual charge remains the same, but the distance between it and the iron has increased so that it produces a smaller effect. The same considerations will explain the effect of the dicarboxylic acids.

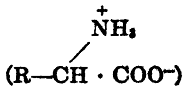
If we apply this reasoning to the phosphoric acids we find that the oxygen atoms of the hydroxyl groups possess a residual charge of $-0.09$. If we consider that one hydroxyl group unites with the iron by means of the usual bond we have two groups left to furnish the additional attraction necessary to prevent the iron from dissociating. These groups are less negative than in the organic acids, but they have the advantage of being on the same atom as the valence bond.

In sulfuric acid we have a different condition. Oxygen and sulfur each possesses a kernel charge of 6+. If we apply the above calculations we find that the oxygen atoms are exactly neutral. Sulfuric acid should then be unable to form undissociated ferric compounds under the conditions of our experiments. Actually we found that it did exhibit a slight effect. This effect is very small in comparison with the $\alpha$-hydroxy acids or the phosphoric acids and may be due to the fact that although oxygen and sulfur each possesses a kernel charge of 6+ they are not exactly equal since oxygen has 4 of its electrons free while sulfur has all of its electrons shared.

If we apply the above considerations to the monocarboxylic amino acids we find that if these acids exist in solution in what may be called the "classical" form

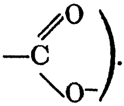


the nitrogen possesses a residual charge of $-0.11$. Then we would expect them to exhibit an effect in our experiments. However, if they exist in the form of the "Zwitter Ion"

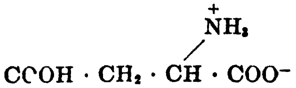


$$(\mathrm{R{-}}\overset{\overset{+}{N}H_3}{\mathrm{CH}}\cdot \mathrm{COO^-})$$

the residual charge on the nitrogen is $+0.89$. They should then exhibit no effect. We saw in the preceding sections that the monocarboxylic amino acids exerted no detectable effect in the concentrations tested. If these amino acids are in solutions that are on the alkaline side of their isoelectric point, then part of the nitrogen would exist as $NH_2$ and should be effective. One cannot work with ferric iron at these reactions, however, because of the insolubility of ferric hydroxide. The effect should be readily detectable with such metals as copper, and certain of the work cited in the introduction indicates that such is the case.

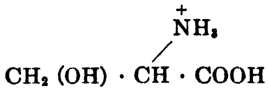
The dicarboxylic amino acids present a different situation. It was found that they produced an effect much greater than the corresponding unsubstituted acids. This does not necessarily mean that the amino group takes any direct part in the union with iron. It will be readily grasped from the above discussion that in our explanation of these undissociated compounds we are considering that two factors are necessary. One of these is a valence.bond, the other is an additional attraction from some other part of the molecule. Then, if other conditions are equal, the amount of iron compound formed is a function of the concentration of the group which unites with iron by means of the bond. If we consider succinic, maleic, and aspartic acids this point will become clear. In each of these acids one carboxyl group furnishes the bond, the other furnishes the additional attraction. Solutions of the same molality, at the same paH, differ only in the concentration of the group which furnishes the bond (*i.e.*  $-\mathrm{C}\begin{smallmatrix}\mathrm{{/\!\!/}O}\\ \mathrm{\diagdown O^-}\end{smallmatrix}$). Since maleic acid is a stronger acid then succinic, when brought to the same paH it will be more ionized and hence offers a greater concentration of the group which unites with iron. If aspartic acid exists as the "Zwitter

Ion" it offers a still greater concentration of this group, for at the paH tested (2.5) it would exist largely as



$$\mathrm{COOH \cdot CH_2 \cdot \overset{\overset{+}{NH_3}}{CH} \cdot COO^-}$$

Serine, however, which is isoelectric at paH 5.7 (65) would exist to a considerable extent as



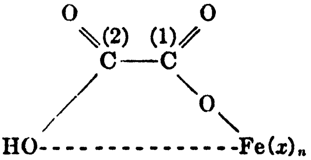
$$\mathrm{CH_2\,(OH) \cdot \overset{\overset{+}{NH_3}}{CH} \cdot COOH}$$

at a paH of 2.5 It may be argued that saying that maleic acid is a stronger acid than succinic acid simply means that due to a difference in the arrangement of its molecule the maleic acid has less attraction for hydrogen ion than succinic acid has. Then by the same reasoning it would have less attraction for a ferric ion, which would offset the advantage of its greater ionization. This reasoning is probably correct, but there is this difference between ferric ion and hydrogen ion. Once the hydrogen ion forms a bond an additional attraction has no effect upon it, but the ferric ion is still susceptible to additional attraction and when this is present it may overcome the lessened attraction due to molecular arrangement. We may point out that the alkali metals would be expected to behave like hydrogen while the other metals would be expected to behave like iron.

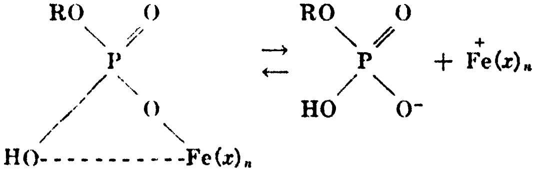
According to these considerations if some group is present to furnish the additional attraction, the strength of an acid is an important factor. This may explain why mandelic acid shows a greater effect than lactic acid and why lactic acid shows a slightly greater effect than α-hydroxybutyric acid. It will also help to explain the marked difference between an α- and a β-hydroxy acid for the latter is a weaker acid in addition to having the hydroxyl farther from the carboxyl group. The same explanation applies to the dicarboxylic acids for the farther apart the two carboxyl groups are placed the weaker is the acid.

It must be pointed out that this additional attraction which we are considering is not entirely a matter of charge. In chloroacetic acid, the chlorine atom possesses a residual negative charge and yet we detected no effect from it in our work. One would expect that chlorine would be less effective in this respect than equally negative nitrogen or oxygen for it has a kernel charge of 7+ in contrast to 5+ and 6+ respectively for the other two. This greater positive kernel charge would offer greater repulsion to the approach of a positively charged ion. This is in agreement with the fact that nitrogen and oxygen form onium compounds much more readily than chlorine does.

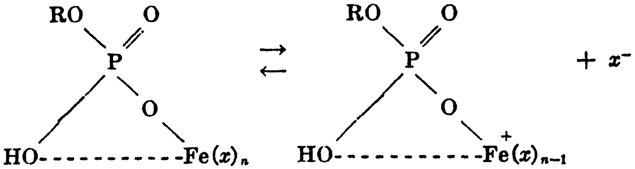
The above considerations are entirely in accord with the results of our migration experiments. If we consider the dicarboxylic acids first, and picture the iron united in the manner suggested, we have



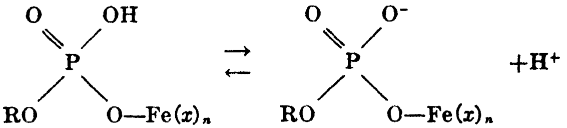
This iron is very slightly dissociated. If carboxyl group (2) ionizes then the iron is present in the anodic form. If the paH of the solution is such as to repress entirely the ionization of carboxyl (2) then the only iron that will move with an electric current is that which dissociates from the above compound and this will be positively charged. Then by varying the paH we should be able to change the direction of migration of the iron. As shown in Table II this can be readily done. If we consider the results given there for glycerophosphoric acid we see that at paH 1.5 the iron is cathodic. According to our interpretation this means that the only iron moving is that which is freed according to the following dissociation.



Or, if we consider that $x$ is different than the glycerophosphoric radical we may have



Now, if the paH is increased to 3.7, the following dissociation is able to take place



and the iron becomes anodic. The reason that the iron in pyrophosphoric acid is anodic at such a low acidity is that the second hydroxyl group of this acid is strongly acidic. It has a $K_a$ value of $1.1 \times 10^{-2}$.

In the case of lactic and mandelic acids the iron is anodic near the neutral point. We interpret this as indicating an ionization of the alcoholic hydroxyl group. Ordinarily this group is very weakly acidic and does not ionize until a much lower acidity is reached. This is in accord with the fact that the oxygen carries a residual negative charge, but if the iron neutralizes this charge it is then easier for the hydrogen to become ionic.

We were unable to obtain anodic iron in glutamic acid solutions. This simply means that the iron is precipitated as the hydroxide before the second carboxyl group can be made to ionize.

We are now in a position to apply the above results to casein. The groupings in the casein molecule which we would expect to unite with iron are the free carboxyl groups and whatever phosphorus is present as a simple ester. Our casein preparation contained 0.81 per cent of phosphorus. Then a 0.94 per cent casein solution is 0.0025 molar with respect to phosphorus. On the basis of Rimington's results (66) we may consider that two-thirds of this phosphorus is present as a simple ester. If we consider that the remaining one-third exerts no effect on our solutions, then the active phosphorus is 0.0017 molar. We may assume that this phosphorus would exert the same effect as an equivalent amount of glycerophosphoric acid. Then from Fig. 4 (Curve II) we find that the phosphorus in 1 cc. of the casein solution would bind 0.08 mg. of iron.

Casein also contains (67) 21.8 per cent glutamic acid, 10.5 per cent hydroxyglutamic acid, and 4.1 per cent aspartic acid. Then a 0.94 per cent solution of casein is 0.0139 molar with respect to glutamic acid, 0.0061 molar with respect to hydroxyglutamic acid, and 0.0029 molar with respect to aspartic acid. If we consider that one carboxyl group of each acid is united in the peptide linkage, then the other must be either free, or present as the amide. The amount of ammonia set free on hydrolysis may be taken as a measure of the amount of amide nitrogen in the protein. This is (67) 1.6 per cent or 0.0088 molar. If we consider that this ammonia is distributed between the glutamic, hydroxyglutamic, and aspartic acids in the ratio of their molalities then 0.0053 molar glutamic, 0.0023 molar hydroxyglutamic, and 0.0011 molar aspartic acids are present as amides. This leaves 0.0086 molar glutamic, 0.0038 molar hydroxyglutamic, and 0.0018 molar aspartic acid existing with one free carboxyl group. Now, if we consider that these groups exert the same effect that the free acids exert we find,[3] from Fig. 5, that for 1 cc. of solution the glutamic acid will bind 0.07 mg. of iron, the hydroxyglutamic acid 0.04 mg. of iron, and the aspartic acid 0.02 mg. of iron. If we add to these the amount bound by the phosphorus we have a total of 0.21 mg. of iron bound. From Fig. 6 we find that 1 cc. of the casein solution actually bound 0.24 mg. of iron. This agreement is not especially good, but it does seem sufficiently good to be quite suggestive.

Now, if the phosphorus is removed from the casein the product so obtained exhibits a behavior almost identical with that of the original product. Rimington (66) has shown that his alkali treatment, which we have followed, in addition to removing the phosphorus also removes all of the amide nitrogen. Then, although we have lost phosphorus we have gained 0.0053 molar glutamic

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[3] Since the concentration of amino acids used to plot the results in Fig. 5 was 0.01 M the amount of iron bound by the respective amino acids at the lower molalities may be obtained with sufficient accuracy by dividing the values given in both ordinate and abscissa by 10.

acid, 0.0023 molar hydroxyglutamic acid, and 0.0011 molar aspartic acid. The above calculation showed that the phosphorus in 1 cc. bound 0.08 mg. of iron. An inspection of Fig. 5 shows that the glutamic acid gained will bind 0.04 mg., the hydroxyglutamic acid 0.03 mg., and the aspartic acid 0.01 mg. of iron for 1 cc. of solution. The net effect of the dephosphorization is thus very small.

1 cc. of a solution containing 1 per cent of dephosphorized casein plus the phosphorus that had been removed from it bound 0.63 mg. of iron. Such a solution should contain all of the phosphorus as the orthophosphate. Then we would expect that 1 cc. of this solution should be equivalent to 1 cc. of a 1 per cent dephosphorized casein solution plus 1 cc. of a 0.0025 molar orthophosphate solution. From Figs. 4 and 6 we find that this should be 0.24 + 0.40 = 0.64 mg. of iron. The agreement is excellent.

If we apply a similar calculation in the case of gelatin the results are not nearly so good as those obtained with casein. This protein contains (67) 5.8 per cent glutamic acid and 3.4 per cent aspartic acid. The solution used contained 1.52 per cent of gelatin. It is 0.0060 molar with respect to glutamic acid and 0.0039 molar with respect to aspartic acid. The ammonia content is 0.4 per cent (67) or 0.0036 molar. If this is divided between the glutamic and aspartic acids in the ratio of their molalities we are left with 0.0038 molar glutamic and 0.0025 molar aspartic acid. From Fig. 5 we can see that for 2 cc. of solution these would bind 0.06 mg. and 0.05 mg. of iron respectively. 2 cc. of the gelatin solution, however, bound 0.33 mg. of iron. Analyses of gelatin have accounted for only 92.4 per cent of its total weight (67). If part of the remainder should prove to be dicarboxylic acids the agreement would be correspondingly improved.

It might be pointed out that the proteins employed in our experiments, casein and gelatin, contain very little or no cystine. It was therefore unnecessary to consider the effect of this amino acid in binding iron.

Brief mention should be made here of results on the migration of iron in various solutions recently reported by Starkenstein (43). A number of his results are in agreement with ours. Others would seem to be in disagreement, although this may be due to the fact that he has not stated the paH of his solutions. Thus he reports that lactic acid forms a complex with iron in which the iron is anodic both in blood and in aqueous solutions. Our results indicate that in aqueous solutions the iron may be cathodic. He also states "Da die einfachen Aminosäuren mit Eisen nur Komplexe bilden, bei denen das Eisen im Kation sitzt, bei dem intermediär gebildeten Ferri-Eiweiszkomplex das Eisen dagegen ein beständiger Teil des Anions ist, können die einfachen Aminosäuren des Eiweiszmoleküls nicht diese Komplexbildung bedingen." On the basis of our work we believe that this statement is quite erroneous.

## SUMMARY.

A study of the substances which may unite with iron to form undissociated compounds is reported. The equilibrium between these substances, ammonium thiocyanate and ferric iron, the stability of the iron compounds to acid and alkali, and the migration of the iron under the influence of an electric current have been investigated.

The data suggest that those substances which possess a particular grouping within their molecule will hold iron as an undissociated compound. The following classes of substances were found to possess this necessary grouping: hydroxymonocarboxylic acids (lactic, gluconic); dicarboxylic acids (oxalic, malonic); hydroxydicarboxylic and hydroxytricarboxylic acids (tartaric, citric); amino acids which are also hydroxy or dicarboxylic acids (aspartic acid, serine); certain inorganic acids (phosphoric, arsenic); certain phosphorus-containing compounds (nucleic acid, glycerophosphoric acid); and certain proteins (casein, gelatin).

A correlation of the amount of iron bound by casein and gelatin with the groupings that are known to occur in their molecules is reported.

An explanation, based on the residual charge of atoms, is suggested for the manner in which the iron may be united.

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# THE MODE OF COMBINATION BETWEEN CERTAIN DYES AND GELATIN GRANULES.*

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In previous papers it was shown by Chapman, Greenberg, and Schmidt (1) and by Rawlins and Schmidt (2) that within the limits of certain acidities the reaction between certain dyes and certain protein solutions takes place in stoichiometric proportions and is therefore chemical in nature. Hewitt (3) concludes from his experiments that salt formation between proteins and dyes takes place and that it is not necessary to postulate a physical adsorption to explain any of the phenomena which he observed. The experiments of Stearn and Stearn (4) indicate that a chemical reaction takes place between dye ions and the oppositely charged ions of protein.

Despite the fundamental statement of Langmuir (5) that "there is no present justification for dividing interatomic (or intermolecular) forces into *physical* and *chemical* forces. It is much more profitable to consider all such forces as strictly chemical in nature. Evaporation, condensation, solution, crystallization, adsorption, surface tension, etc., should all be regarded as typical chemical phenomena," there is still a considerable number of workers who attempt to distinguish between adsorption and chemical combination.[1] If we assume for the moment that all interatomic reactions are chemical (as Langmuir does) we still must admit that

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[1] For the various theories which have been proposed to explain the phenomenon of dyeing and staining see Zacharias, P., Die Theorie der Farbevorgänge, Berlin, 195 (1908), and Pelet-Jolivet, L., Die Theorie des Farbeprozesses, Dresden, 1 (1910).

some of these reactions involve primary valences of the atoms (or the molecules). The existence of primary valences may be demonstrated by demonstrating stoichiometric proportions. Other of these reactions involve secondary valences and forces which are more generally distributed throughout the space about the atom. These are manifested by solution, crystallization forces, etc. They may lack the quantitative aspect which reactions of primary valence have. We have considered it of interest to determine whether the reaction which takes place between gelatin granules and certan dyes belongs to the class of primary valence reactions or to the quantitatively less definite group of reactions such as adsorption, etc.

Among the arguments advanced in favor of adsorption is that combination takes place in accord with the adsorption isotherm:

$$\frac{x}{m} = KC^{\frac{1}{n}}$$

Thus Grollman (6) concludes from his experiments that the combination between phenol red and certain proteins is a phenomenon of adsorption. An examination of Grollman's data shows that in the experiments with gelatin and phenol red the solution of dye used was quite dilute and the amount of dye which was bound by the gelatin even at favorable acidities was but a small fraction of the amount which gelatin is capable of binding. Phenol red is a weak acid. The value for $Ka$ is $1.2 \times 10^{-8}$. It is therefore not unexpected that at acidities in which the maximum number of the basic groups of gelatin come into play the amount of phenol red which is bound should be less than that at acidities closer to the isoelectric point of gelatin.

In our previous experiments on the mode of combination which takes place between certain proteins and dyes, protein solutions were employed. The system was homogenous. A few experiments were also carried out with gelatin granules. It was pointed out that time was a very important factor in attaining equilibrium in a heterogenous system of dye and protein. Due to the fact that in the staining of protoplasm and the dyeing of fabrics the system is not homogenous, we have considered it desirable to extend our studies to heterogenous systems of protein and dye. In this paper comparisons are made between the nature of the reactions which take place between certain dyes and gelatin solutions and gelatin granules.

Gelatin was chosen for the reason that it is unique in that it can exist in solution or in particulate form under the same conditions of experimentation. The materials which were employed were the same as those which were previously described. The gelatin was isoelectric. It contained about 10 per cent of moisture. It was used in a concentration of about 0.18 per cent. The technique consisted in weighing out two samples of gelatin and adding distilled water to a volume of 50 cc. One of the two samples of gelatin was dissolved with the aid of heat while the other remained in the form of granules. Both samples were now placed in a thermostat for several hours. In the concentrations employed the gelatin solution did not set to a gel. The gelatin granules did not dissolve to any appreciable extent except those which were heated to 28° and 30°. The amount which was dissolved at these temperatures was not large. A definite amount of 0.1 N hydrochloric acid was now added to all samples of gelatin. After standing a short time this was followed by addition of the desired amount of dye solution which had previously been brought to the same temperature as the flasks which contained the gelatin. Increasing amounts of dye were added to successive flasks containing the same amounts of gelatin in the form of granules and in solution. The dye was added with rapid stirring and this was continued for about a minute. The mixture was then allowed to stand for the desired length of time.

The amount of dye which was present in the supernatant solution was determined colorimetrically after the filtering off of the gelatin-dye granules or, in those cases where gelatin solutions were employed, the precipitates. Hydrochloric acid was added to the standard dye solution in order to eliminate the possible effect of these substances on the color of the dye. It was quite easy to match the colors of the unknown dye solution against the standard dye solution except over the range which we shall temporarily designate the "protective colloidal region." In this region the colors did not match. In the protective colloidal region the protein-dye compound did not precipitate and it was not until a further amount of dye was added that precipitation of the dye-protein compound took place. This protective colloidal effect accounts for the S-shaped curves shown in Figs. 1 to 7. In order to obtain proper color readings in this region the following method

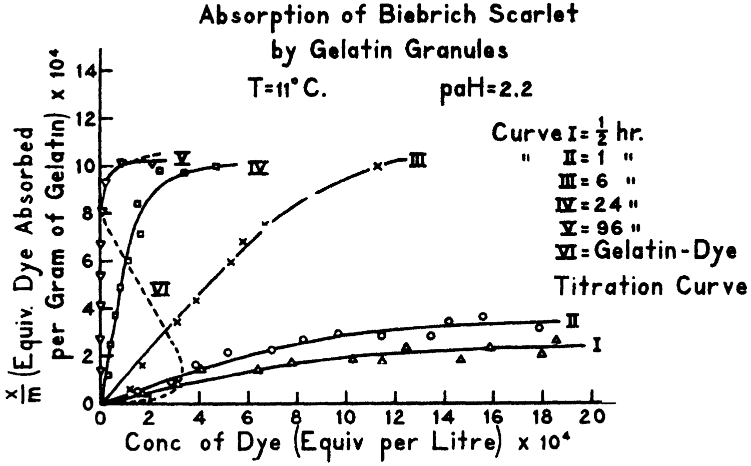


FIG. 1.

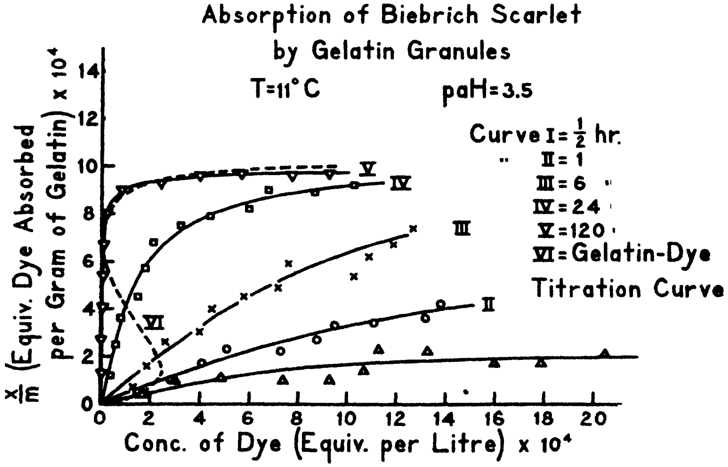


FIG. 2.

was employed: When on titrating gelatin solutions with dye the solution remained clear and no precipitate formed it was assumed that the dye was in solution (although in part or entirely combined with gelatin) and this solution was taken as the standard for the colorimetric estimation of the dye in those solutions in which a precipitate did form but in which the protective colloidal effect still existed. The experiments were carried out not only at different temperatures but also at different acidities.

The data which were obtained in the experiments with Biebrich scarlet and gelatin are graphically shown in Figs. 1 to 3. An inspection of the curves shows that the amount of dye absorbed by the gelatin granules is, within certain limits, dependent upon the time during which the gelatin granules are in contact with the dye. Thus in Fig. 1 the amount of dye which is taken up by the gelatin granules during a period of 1 hour is about one-third of that which the gelatin granules absorb during a period of 96 hours. A comparison of Curves I and II in Fig. 1 with the corresponding curves in Fig 3 shows that at 21° more dye is absorbed by the gelatin granules during the half hour and 1 hour periods than is taken up during corresponding periods of time at 11°. A comparison of Curves III in the same figures indicates that it is necessary that a greater concentration of dye be present in the solution at 11° than at 21° in order that the amount of dye absorbed by the gelatin granules shall be the same. The final amount of dye absorbed by the gelatin granules at paH 2.2 is

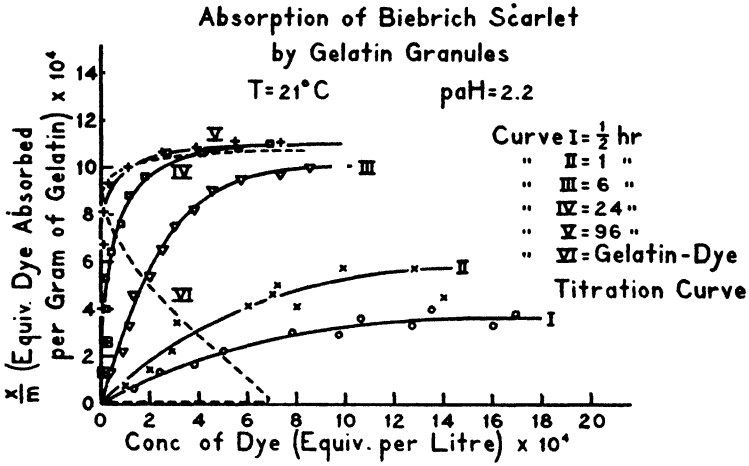


FIG. 3.

essentially the same at 11° and 21° when the time during which the gelatin granules are in contact with the dye is extended to a

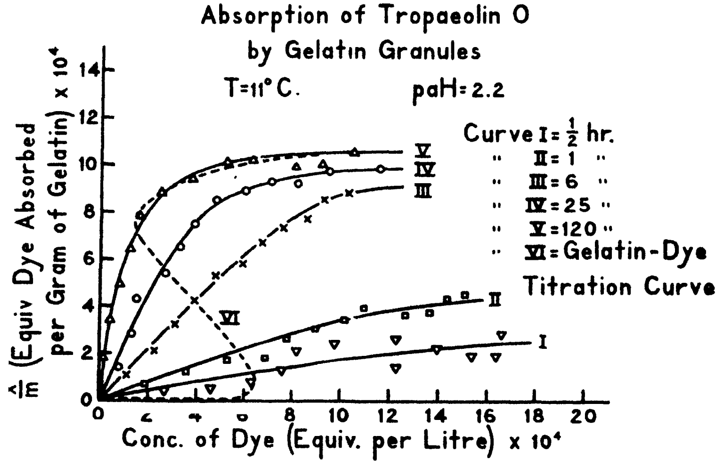


FIG. 4.

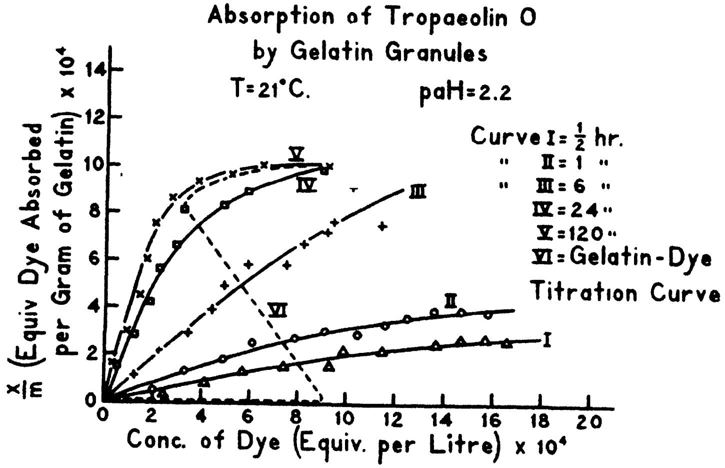


FIG. 5.

period of 96 hours. The amount of dye absorbed at paH 3.5 is a little less than the amount which the gelatin granules took up at paH 2.2. In both cases the temperature was 11°. The dotted lines in Figs. 1 to 3 represent the gelatin-dye titration curves obtained with gelatin solutions. The S shape form of these curves

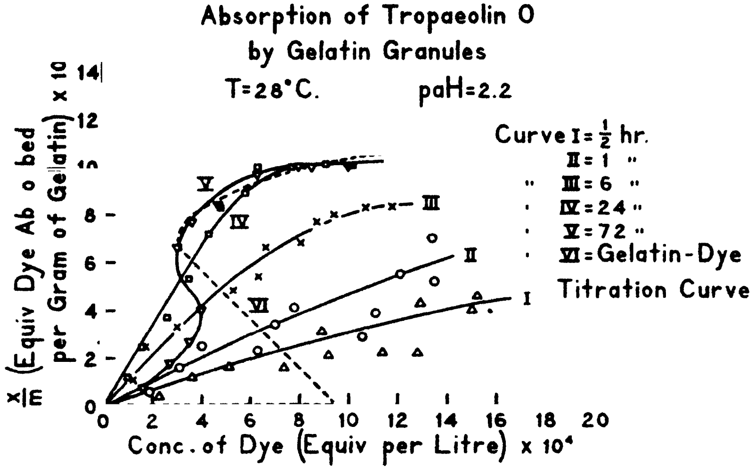
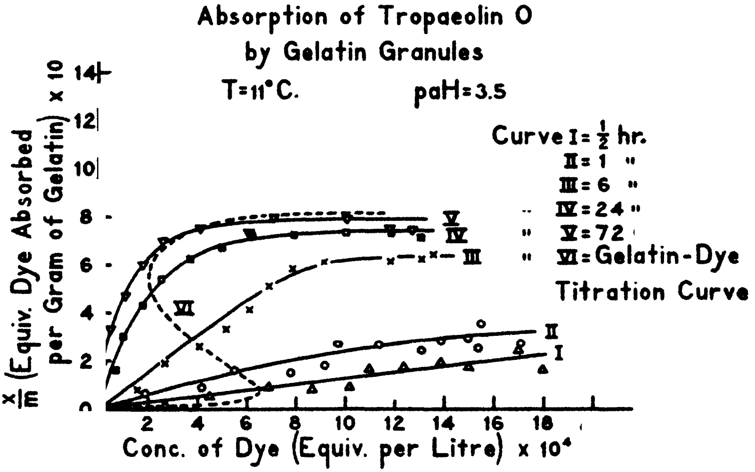


FIG. 6.



indicates a protective colloidal effect. The significance of this will be discussed later. It is significant that the maximum amount of Biebrich scarlet which was absorbed by the gelatin granules when the time during which the gelatin granules were in contact with the dye was extended to a period of 96 or 120 hours was the same at the same acidities as the amount which was combined with gelatin when the latter was in solution. This is indicative of the fact that both reactions are the same and indicate chemical combination.

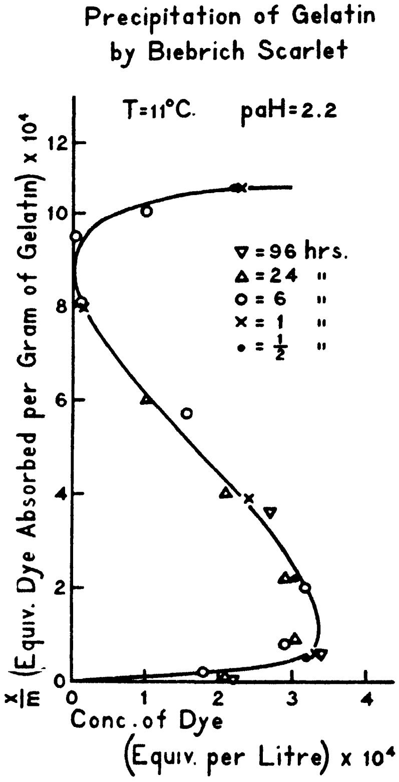


FIG. 8.

The experimental data obtained with tropeolin O and gelatin are graphically represented in Figs. 4 to 7. The data in a general sense corroborate the statements which have been made relative to Biebrich scarlet and gelatin. A comparison of Curves II in Figs. 4 and 5 shows that the amount of dye which was absorbed at 21° was not very different from the amount which was absorbed at 11°. However, the effect of temperature in accelerating the equilibrium between dye and gelatin granules is shown by Curve II of Fig. 6. The S shape form of Curve V of Fig. 6 indicates that due to the higher temperature employed some gelatin dissolved during the 72 hour period and hence the protective colloidal effect enters. A comparison of Curve V in Fig. 7 with Curve V in each of Figs. 4, 5, and 6 shows that less dye is absorbed by the gelatin granules at paH 3.5 than at paH 2.2.

The S shape form of the dotted curve representing the dye titration curve with gelatin in solution in each of Figs. 4 to 7 is indicative of protective colloidal effect. From each of these figures it is apparent that the maximum amount of dye which is taken up by the gelatin granules at the maximum times indicated is essentially the same as that with which gelatin combined when the latter was in solution. In plotting the S-shaped curves we have not included the experimental points which form the basis of the curves. The reason for this lies in the difficulty of plotting the many experimental points which form the bases of these curves. In order to show how the S-shaped curves were obtained we have plotted in Fig. 8 the experimental data for the S-shaped curve shown in Fig. 1. Except within the protective colloidal region the experimental points were found to fall somewhat better along the plotted S-shaped curves of the gelatin solution titrations than the experimental points fall on the gelatin granules-dyes curves. It was found that within the limits of ½ and 120 hours time appeared to have no effect on the gelatin solution-dye titration curves. This is of interest since it indicates that no decomposition of gelatin took place and that in whatever way the gelatin solution may have changed on standing, *i.e.* the change from molecules to micelles or aggregates, such a change exerted no effect on the combining capacity of gelatin for the dyes used.

In Fig. 9 the data which were obtained with Biebrich scarlet and gelatin granules and represented graphically in Fig. 2 have been plotted on a logarithmic scale. Similar data obtained with tropeolin O and gelatin granules and represented in Fig. 4 have been plotted logarithmically in Fig. 10. We have also plotted the absorption data represented in Figs. 2 and 4 respectively on a negative logarithmic scale in Figs. 11 and 12 respectively. This method of plotting was employed by Grollman (6) and from the

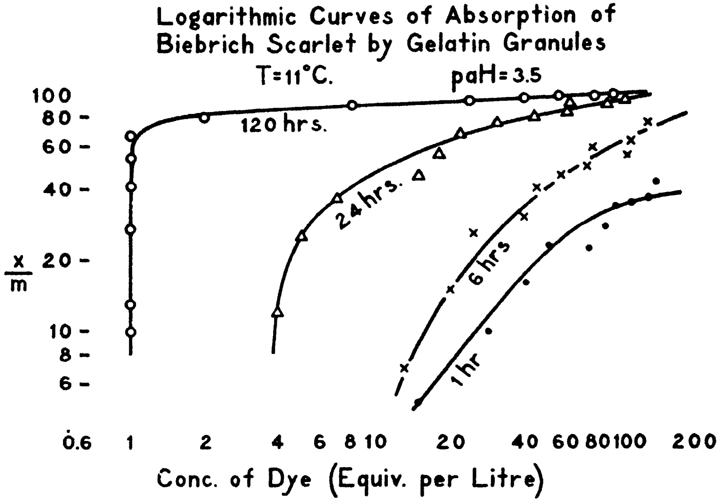


Fig. 9.

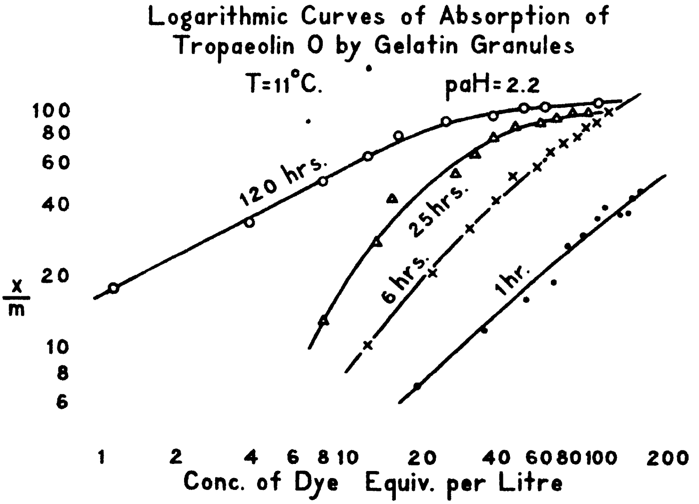


Fig. 10.

fact that his curves were straight lines he interpreted this to indicate that the taking up of dye by gelatin granules is an adsorption phenomenon. An inspection of the curves in Figs. 9 and 10 indicates that the curves which approach most nearly to a straight line are those which represent experiments in which the gelatin granules were permitted to remain in contact with the dye solutions for relatively short periods of time. However, the curves representing 24, 25, and 120 hour periods of time deviate considerably from straight lines. The same thing is shown by the curves which are represented in Figs 11 and 12. The facts show that when the amounts of Biebrich scarlet and tropeolin O which are absorbed by gelatin granules are large and represent the maximum amount with which the gelatin can combine at the particular acidities employed the relation between the amount of dye which is taken up by the gelatin granules and the concentration of dye employed cannot be expressed by the adsorption formula. Moreover, since the maximum amount of Biebrich scarlet and of tropeolin O which gelatin granules absorbed at the acidities employed in our experiments was the same as that with which gelatin when in solution was found to combine, and since it has been shown that the latter process occurs in stoichiometric proportions and is therefore chemical in nature, it follows that the absorption of the

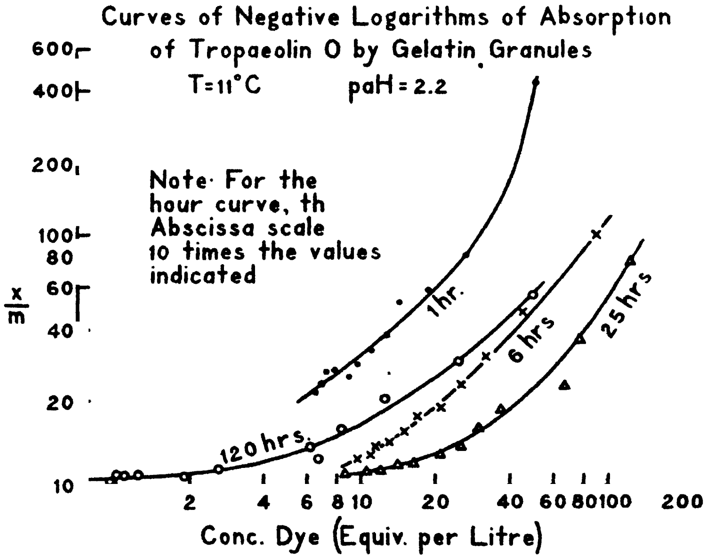


FIG. 11.

above dyes by gelatin granules is also dependent upon primary chemical forces.

The data further show that surface appears to play no part in the final end-point of the reaction since it has been shown that changing the surface of gelatin from that of granules to that of molecules or aggregates did not affect the amount of dye with which the gelatin was able to combine. The apparent effect of increasing the size of the gelatin unit was to increase the distance through which the dye had to travel before it could reach and combine with the gelatin of the innermost part of the granules.

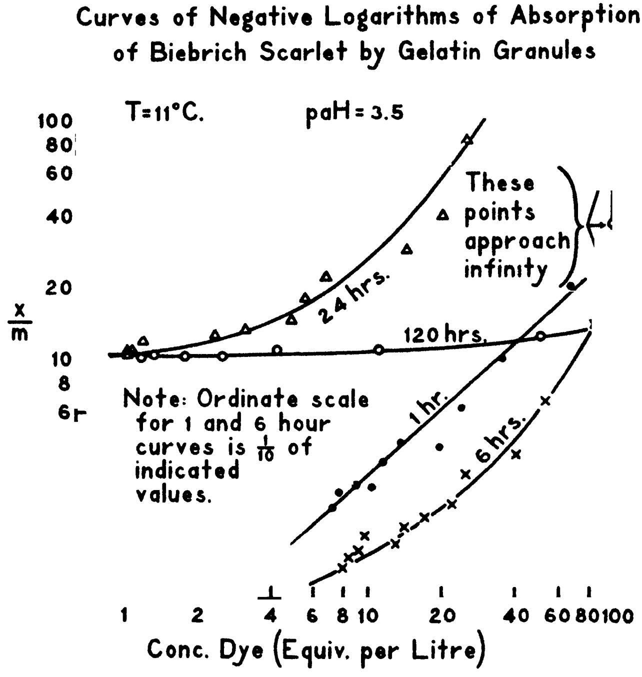

Curves of Negative Logarithms of Absorption of Biebrich Scarlet by Gelatin Granules

FIG. 12.

Attention must be called to the fact that in our dye absorption experiments the effect of the Donnan membrane equilibrium between dye and gelatin granules was not considered. This factor probably enters not only in our experiments but also in the experiments of others who have worked with similar systems. However, this factor probably does not enter to any appreciable extent in such mixtures in which, at the acidities employed, the maximum amount of dye with which the gelatin can combine is taken up by the gelatin particles. This is due to the fact that the gelatin dye compounds form insoluble precipitates.

It is seen from the dotted curves in Figs. 1 to 7 that although the dyes which were employed form insoluble compounds with gelatin the precipitates do not appear until a certain concentration of dye had been employed. This is not a phenomenon analogous to supersaturation. While our experiments do not throw definite light upon this subject, a tentative hypothesis may be advanced. It is conceivable that the solubility of the gelatin is dependent upon an orientation of the acidic or basic groups of this protein to water. A few of these groups may be neutralized by combination with dye and still enough of them will be left to permit the gelatin dye compound to remain in solution. On continued addition of dye a point will be reached when there will not be a sufficient number of these groups left to permit the gelatin to remain in solution. Precipitation will then occur. At this point the maximum amount of dye with which the gelatin can combine will not necessarily have been taken up by the gelatin molecules. Some of the basic or acidic groups will still be free. On addition of further amounts of dye more dye will combine with gelatin until at the particular paH employed all of the groups will have been neutralized.

## SUMMARY.

1. Experiments have been carried out to determine the effects of concentration of dye, temperature, time, and acidity on the amounts of Biebrich scarlet and of tropeolin O which gelatin granules can take up.

2. It is shown that the amounts of dye which gelatin granules absorb are dependent on the time during which the granules are in contact with the dye, the acidity of the solution, and the concentration of dye employed.

3. The experiments show that at the acidities indicated the maximum amounts of the dyes which were taken up by gelatin granules at the maximum periods of time employed were the same as those which were found to combine with gelatin when the latter was in solution.

4. From this fact and the fact that the so called adsorption curves of gelatin with Biebrich scarlet and tropeolin O at the longer periods of time when plotted logarithmically are not straight lines, it is concluded that the phenomenon is not one of adsorption but is dependent upon primary chemical forces.

5. A tentative hypothesis is advanced to explain the S-shaped curves (*i.e.* the protective colloidal effect) which were obtained when varying amounts of dye were added to gelatin solutions.

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# THE APPARENT DISSOCIATION CONSTANTS OF ARGININE AND OF LYSINE AND THE APPARENT HEATS OF IONIZATION OF CERTAIN AMINO ACIDS.*

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In this paper the apparent dissociation constants of arginine and of lysine at 25° and the apparent dissociation constants of arginine, lysine, histidine, aspartic acid, and glutamic acid at 0° are reported. We have made use of these data together with previously published data for the apparent dissociation constants of histidine, aspartic acid, and glutamic acid for the purpose of calculating the apparent heats of ionization of these amino acids.

The technique employed for obtaining the titration curves was the same as that which has been described by Kirk and Schmidt (1) except that for the purpose of carrying out hydrogen ion activity measurements at 0° the calomel electrode, potassium chloride solution, and the hydrogen electrode vessel were immersed in an ice-water bath. A modified electrode vessel suitable for immersion in water was used. Uniform temperature was obtained by rapid stirring. For the purpose of determining the value of the calomel electrode at 0° the electromotive force of a hydrogen electrode immersed in a 0.01 N hydrochloric acid plus 0.09 N potassium chloride solution was determined. The relation between the measured electromotive force, the electromotive force of the calomel cell, and the hydrogen ion activity at 0° is given by the equation

$$E_0 = E_0' - \log \frac{1}{(\mathrm{H}^+)_0}$$

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where $E_0$ = the measured E.M.F. at 0°, $E_0'$ = the E.M.F. assigned to the calomel cell at 0° when $(H^+)_0 = 1$ and $(H^+)_0$ = the hydrogen ion activity at 0°. The value of $(H^+)_0$ was computed from the equation

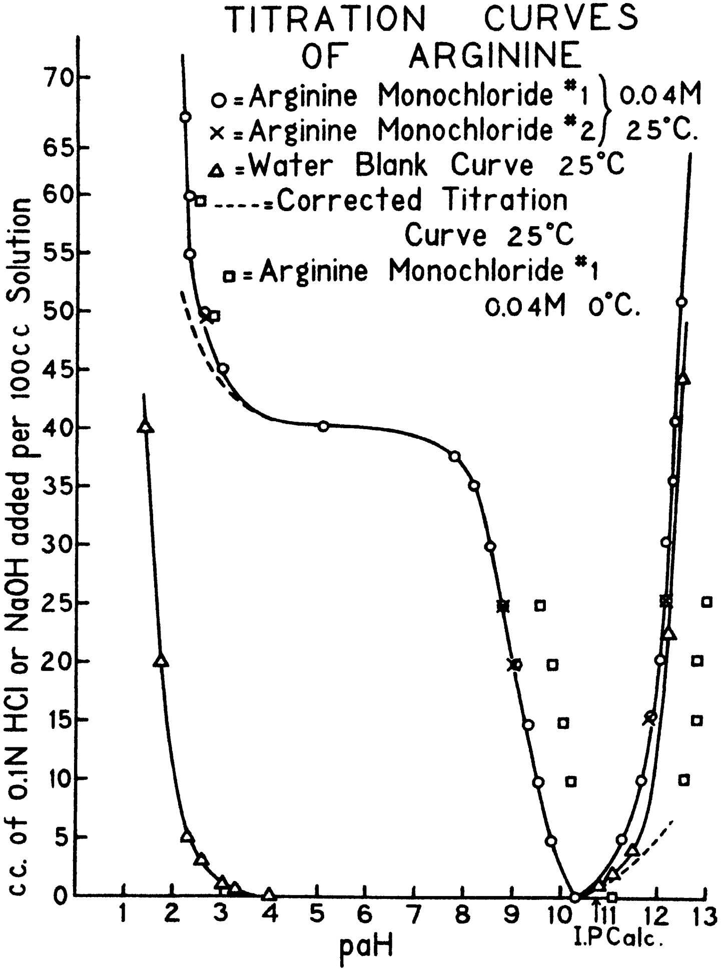

Fig. 1.

$$(H^+)_0 = C\gamma_0$$

where $C$ = concentration and $\gamma_0$ = the activity coefficient of the hydrogen ion at 0°. The value of $\gamma_0$ was taken as 0.907 (2). The value of $E_0'$ was found to be 0.2916 volt. The dissociation constant for water at 0° was taken as $0.114 \times 10^{-14}$.

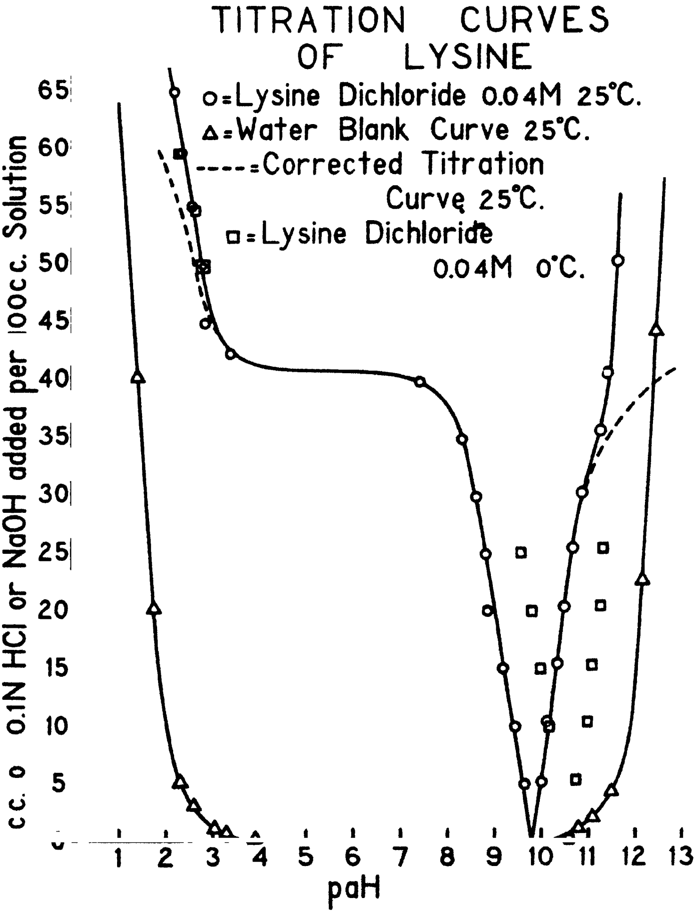

Fig. 2.

Arginine monochloride and lysine dichloride were isolated by the method of electrical transport described by Foster and Schmidt (3) and more recently elaborated by Cox, King, and Berg (4). A second preparation was obtained from the Eastman Kodak

TABLE I.

*Titration Data for Aspartic Acid, Glutamic Acid, and Histidine Dichloride at 0°.*

All amino acid solutions were 0.04 M.

| Amino acid. | 0.1 N NaOH per 100 cc. of solution. | 0.1 N HCl per 100 cc. of solution. | E.M.F. | paH | Apparent dissociation constants. | pI |
|---|---|---|---|---|---|---|
| | *cc.* | *cc.* | *volt* | | | |
| Aspartic acid. | 10.1 | | | | | |
| | 16.2 | | 0.4865 | 3.596 | | |
| | 20.2 | | 0.4989 | 3.824 | | |
| | 25.3 | | 0.5092 | 4.015 | $K'_{a_1} = 1.7 \times 10^{-4}$ | |
| | 50.5 | | 0.8278 | 9.892 | | |
| | 60.6 | | 0.8492 | 10.289 | $K'_{a_2} = 4.9 \times 10^{-11}$ | |
| | | 11.9 | 0.4300 | 2.554 | | |
| | | 16.8 | 0.4222 | 2.409 | | |
| | | 21.8 | 0.4107 | 2.198 | | |
| | | 26.7 | 0.4043 | 2.078 | $K'_{b_1} = 1.17 \times 10^{-13}$ | 2.89 |
| Glutamic acid. | 10.1 | | 0.4916 | 3.691 | | |
| | 16.2 | | 0.5060 | 3.957 | | |
| | 20.2 | | 0.5173 | 4.164 | $K'_{a_1} = 7.25 \times 10^{-5}$ | |
| | 25.3 | | | | | |
| | 50.5 | | 0.8216 | 9.778 | | |
| | 60.6 | | 0.8458 | 10.226 | $K'_{a_2} = 6.0 \times 10^{-11}$ | |
| | | 11.9 | 0.4315 | 2.581 | | |
| | | 16.8 | 0.4284 | 2.524 | | |
| | | 21.8 | 0.4189 | 2.349 | | |
| | | 26.7 | 0.4108 | 2.199 | $K'_{b_1} = 1.95 \times 10^{-13}$ | 3.19 |
| Histidine dichloride. | 10.1 | | 0.7977 | 9.338 | | |
| | 15.1 | | 0.8010 | 9.399 | | |
| | 20.2 | | 0.8222 | 9.790 | | |
| | 25.2 | | 0.8317 | 9.966 | $K'_{a} = 1.78 \times 10^{-10}$ | |
| | | 9.9 | 0.6717 | 7.015 | | |
| | | 14.8 | 0.6535 | 6.678 | | |
| | | 19.8 | 0.6441 | 6.505 | | |
| | | 24.8 | 0.6339 | 6.315 | $K'_{b_1} = 3.63 \times 10^{-9}$ | |
| | | 49.5 | 0.4307 | 2.567 | | |
| | | 59.4 | 0.4121 | 2.223 | $K'_{b_2} = 9.12 \times 10^{-14}$ | 8.12 |

Company. All products were recrystallized two to three times. Amino nitrogen determinations gave within the limits of error theoretical values. The glutamic acid and the histidine dichloride were the identical products which have been previously described (1, 5). The aspartic acid was a Kahlbaum preparation. It was recrystallized three times.

The titration curve of arginine at 25° is shown in Fig. 1 and that of lysine at the same temperature is represented in Fig. 2. For arginine the following values were obtained for the dissociation constants at 25°: $K'_{a_1} = 3.32 \times 10^{-13}$, $K'_{b_1} = 1.10 \times 10^{-5}$, $K'_{b_2} = 1.05 \times 10^{-12}$, and pI = 10.75. The values for lysine at this temperature are: $K'_{a_1} = 2.95 \times 10^{-11}$, $K'_{b_1} = 0.89 \times 10^{-5}$, $K'_{b_2} = 1.52 \times 10^{-12}$, and pI = 9.74. Although the complete titration curves of arginine and of lysine at 0° are not shown a sufficient number of experimental points are given in each figure so that the apparent dissociation constants of the respective amino acid at this temperature may be calculated. On the basis of the plotted points shown in Fig. 1 the apparent dissociation constants of arginine at 0° are: $K'_{a_1} = 4.9 \times 10^{-14}$, $K'_{b_1} = 7.09 \times 10^{-6}$, $K'_{b_2} = 2 \times 10^{-13}$, and pI = 11.55. The apparent dissociation constants of lysine at 0° calculated from Fig. 2 are: $K'_{a_1} = 4.9 \times 10^{-12}$, $K'_{b_1} = 7.4 \times 10^{-6}$, $K'_{b_2} = 1.82 \times 10^{-13}$, and pI = 10.56. Table I gives the experimental data from which the apparent dissociation constants of aspartic acid, glutamic acid, and histidine at 0° (also given in the table) have been calculated.

Our values for the dissociation constants of lysine at 25° agree quite well with those which have been reported by Simms (6). He found $K'_a = 3.4 \times 10^{-11}$, $K'_{b_1} = 1.11 \times 10^{-5}$, and $K'_{b_2} = 1.11 \times 10^{-12}$. In the case of arginine the agreement with the values for the constants at 25° found by Simms (6) is not very good.[1] The following are his recalculated values: $K'_a = 2.29 \times 10^{-10}$, $K'_{b_1} = 1.43 \times 10^{-6}$, and $K'_{b_2} = 1.97 \times 10^{-12}$. The discrepancy lies in the values for $K'_a$ and $K'_{b_1}$. Our values for arginine are in better agreement with the data reported by Hunter and Borsook (7). Their recalculated data at 20° (8) yield the following values: $K'_a = 1.4 \times 10^{-13}$, $K'_{b_1} = 8.8 \times 10^{-6}$, and $K'_{b_2} = 1.07 \times 10^{-12}$. A comparison of the values for the dissociation constants at 0° and 25° shows that the temperature influence is greatest on the smaller apparent dissociation constant values and least on the

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[1] In a private communication dated March 20, 1930, Dr. Simms states that his published values for the apparent dissociation constants of arginine at 25° are in error and accepts the values reported by us in this paper.

larger values. This is in agreement with the well known fact that the relative effect of temperature on the dissociation constants of highly ionized compounds is less than on compounds which are dissociated to a slight extent.

Using the data for the dissociation constants at 0° and 25° of the amino acids given in Table II it is possible to calculate their apparent heats of ionization Data for glycine, alanine, glycylglycine and glycylalanine obtained by this method of calculation have recently been published by Branch and Miyamoto (9). Meyerhof (10) had previously determined the heat of ionization of glycine and Adair, Cordero, and Shen (11) have reported the heat of ionization of hemoglobin.

For the purpose of calculating the apparent heat of ionization of the amino acids reported in this paper we have made use of the equation

$$-\Delta H = \frac{d(R \ln K)}{d\left(\frac{1}{T}\right)} = \frac{4.5787\, d \log K}{d\left(\frac{1}{T}\right)}$$

where $T$ = the absolute temperature, $R$ = the gas constant in calories, $K$ = the true dissociation constant, and $\Delta H$ = the heat of ionization or the change in heat content of the solution as the result of ionization. In using this equation the following assumptions are made: (*a*) that the equation holds when the values for the apparent dissociation constants of the amino acids are used instead of the true dissociation constants; (*b*) that the increment $\Delta H$ is constant over the temperature range of 0–25°. This is equivalent to assuming that

$$\frac{\Delta \log K}{\left(\frac{1}{T}\right)} = \frac{d \log K}{d\left(\frac{1}{T}\right)}$$

between 0° and 25°. These assumptions are the same as those which were made by Branch and Miyamoto (9). Although the validity of these assumptions is open to question the errors which are thus introduced are probably not much greater than certain other errors inherent in obtaining the values for the apparent dissociation constants.

TABLE II.

*Apparent Heats of Ionization.*

| Amino acid. | Temperature. | pK' | $pK'_{(0°)} - pK'_{(25°)}$ | $\Delta H'$ |
|---|---|---|---|---|
| | *°C.* | | | *calories* |
| Arginine. | 0 | $pK'_a = 13.31$ | | |
| | 25 | $pK'_a = 12.48$ | 0.83 | 12400 |
| | 0 | $pK'_{b_1} = 5.15$ | | |
| | 25 | $pK'_{b_1} = 4.96$ | 0.19 | 2830 |
| | 0 | $pK'_{b_2} = 12.70$ | | |
| | 25 | $pK'_{b_2} = 11.99$ | 0.71 | 10600 |
| Histidine. | 0 | $pK'_a = 9.75$ | | |
| | 25 | $pK'_a = 9.12$ | 0.63 | 9400 |
| | 0 | $pK'_{b_1} = 8.44$ | | |
| | 25 | $pK'_{b_1} = 7.96$ | 0.48 | 7200 |
| | 0 | $pK'_{b_2} = 13.04$ | | |
| | 25 | $pK'_{b_2} = 12.18$ | 0.86 | 12800 |
| Lysine. | 0 | $pK'_a = 11.31$ | | |
| | 25 | $pK'_a = 10.53$ | 0.78 | 11600 |
| | 0 | $pK'_{b_1} = 5.13$ | | |
| | 25 | $pK'_{b_1} = 5.05$ | 0.08 | 1200 |
| | 0 | $pK'_{b_2} = 12.74$ | | |
| | 25 | $pK'_{b_2} = 11.82$ | 0.92 | 13100 |
| Aspartic acid. | 0 | $pK'_{a_1} = 3.77$ | | |
| | 30 | $pK_{a_1} = 3.63$ | 0.14 | 1760 |
| | 0 | $pK'_{a_2} = 10.31$ | | |
| | 30 | $pK'_{a_2} = 9.47$ | 0.84 | 10500 |
| | 0 | $pK'_b = 12.93$ | | |
| | 30 | $pK'_b = 12.09$ | 0.84 | 10500 |
| Glutamic acid. | 0 | $pK'_{a_1} = 4.14$ | | |
| | 25 | $pK_{a_1} = 4.07$ | 0.07 | 1040 |
| | 0 | $pK'_{a_2} = 10.22$ | | |
| | 25 | $pK'_{a_2} = 9.47$ | 0.75 | 11200 |
| | 0 | $pK_b = 12.71$ | | |
| | 25 | $pK_b = 11.90$ | 0.80 | 12100 |

The values for the apparent heats of ionization $\Delta H'$ of the amino acids are given in Table II. In the calculations we have used the values for the constants of arginine, lysine, histidine, aspartic acid, and glutamic acid experimentally determined by us and reported in this paper. The constants for histidine at 25° were those previously reported by ourselves (5). The glutamic acid constants used were those which were determined by Simms (6, 8). If the constants for glutamic acid at 25° reported by Kirk and Schmidt (1) are used instead of Simms' values an irrational value for $\triangle H'$ corresponding to $\mathrm{p}K'_{a_1}$ is obtained. This is due to the fact that the value for $\triangle H'$ corresponding to $K'_{a_1}$ is small and a small error in the value for $K'_{a_1}$ is magnified in the value for $\triangle H'$. On the other hand, the value for $\triangle H'$ of glutamic acid corresponding to $\mathrm{p}K'_{b_2}$ when similarly calculated are approximately 10 per cent greater than the value given in Table II This probably represents the error inherent in determining the values for $\triangle H'$. The dissociation constants for aspartic acid at 30° were those which have been reported by Levene and Simms (12).

An inspection of Table II shows that the order of magnitude of the values for $\triangle H'$ corresponding to $K'_{a_1}$ and $K'_{b_2}$ of arginine and of lysine and $K'_{a_2}$ and $K'_{b_2}$ of aspartic acid and of glutamic acid are approximately the same and are not very different from the values for $\triangle H'$ reported for glycine and for alanine by Branch and Miyamoto (9). The values for $\triangle H'$ corresponding to $K'_{a_1}$ of aspartic acid and of glutamic acid and to $K'_{b_1}$ of arginine and of lysine are small and roughly of the same order of magnitude. The value for $K'_{b_1}$ of histidine occupies a position intermediate between the values for $K'_{b_1}$ of arginine and its own value for $K'_{b_2}$ and this is reflected in the value for $\triangle H'$ corresponding to $K'_{b_1}$ of histidine which is intermediate between the values for $\triangle H'$ corresponding to $K'_{b_1}$ of arginine and to $K'_{b_2}$ of histidine.

## SUMMARY.

1. The apparent dissociation constants at 25° have been determined for arginine and for lysine and at 0° for arginine, histidine, lysine, aspartic acid, and glutamic acid.

2. On the basis of these data and certain others cited in the text the apparent heats of ionization of arginine, histidine, lysine, aspartic acid, and glutamic acid have been calculated.

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# ENZYME PURIFICATION: FURTHER EXPERIMENTS WITH PANCREATIC AMYLASE.*

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A consideration of the results of our recent experiments upon the use of adsorption in the purification of pancreatic amylase (1) indicated that it would be advantageous to continue the intensive study of this method of purification and also to determine the best way of following adsorption with precipitation to obtain the final enzyme preparation as a dry solid, as in our previous method (2).

The general plan of the work was to study the factors involved in: obtaining highly active enzyme solutions for the adsorption; adsorption and extraction of the amylase from its adsorbent; and precipitation and drying of the solid products.

## EXPERIMENTAL.

The amylase activity was measured at each step so that the total recovery and the degree of purification could be followed quantitatively throughout. In solutions containing glycerol, it was not possible to compare the amylase activities on the basis of total solids and therefore the basis of activity per mg. of nitrogen was chosen.

The conditions for measuring the activity of the enzyme were kept constant in all the experiments. These were half hour hydrolyses at 40° of 2 per cent starch dispersions containing 0.02 M sodium chloride and 0.01 M phosphate, made up by the use of equimolar solutions of mono- and disodium phosphates in the proportions necessary to give a hydrogen ion activity of pH 7.1 (3).

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The solutions and suspensions obtained in the process of purification were diluted to a definite volume of the same final salt concentration and hydrogen ion activity as the starch dispersion to which they were added in suitable portions. The maltose formed was taken as the measure of the amylase activity. The small amounts of alumina introduced in the suspensions were found, by suitable blank determinations, not to interfere with the measurements of the amylase activity.

The hydrogen ion activities of all solutions and suspensions were measured electrometrically, with a saturated calomel electrode and a hydrogen electrode of the type described by Wilson and Kern (4). It was found practicable to apply to the dilute glycerol and alcohol solutions sometimes used, E.M.F. measurements as usually made in determinations of hydrogen ion activities in aqueous solutions. Steady E.M.F. readings were obtained, and these solutions could readily be reproduced or titrated to the same values. The E.M.F. measurements were calculated to pH values in the usual way although it was recognized that these values might not bear the same relation to the reaction of the solutions as they would in aqueous solutions containing no glycerol or alcohol.

The finding that 0.01 M phosphate did not interfere with or apparently influence the adsorption of this amylase by alumina gel or with its subsequent extraction from the alumina gel by which it had been adsorbed, made possible its use for the control of the reaction of the solutions and suspensions throughout the process.

All precautions which have been found necessary in working with this amylase such as keeping solutions and reagents ice-cold and guarding against the influence of light and fumes were observed. All reagents were highly purified.

*Solution for Adsorption of Pancreatic Amylase by Alumina Gel.*—Experimental study of the extraction of pancreatin, in which the influence of time, temperature, concentration of pancreatin, and the nature of the solvent was each investigated, led us to adopt the use of 87 per cent glycerol recommended by Willstätter *et al.* (5), for the extraction; this was followed by the removal of water-insoluble inert material from the extract by dilution with 5 volumes of water containing a phosphate mixture so adjusted that the final concentration was 0.01 M and the reaction pH 5.8. The disadvantages (1) of the presence of glycerol in the later stages of purification were overcome by the fact that the amylase may be effectively separated from the glycerol by adsorption by alumina gel.

*Adsorption As Influenced by Different Conditions.*—Among the factors which were found to influence markedly the adsorption of the amylase were: the reaction of the solution, the concentration of alcohol, and the relative concentrations of enzyme and alumina. The influence of each of these factors was studied.

The optimal reaction, calculated as previously mentioned, for the adsorption and purification of the amylase appeared to differ in the presence of the different percentages of alcohol: pH 5.8 for 0 alcohol, pH 5.7 for 10 per cent, pH 5.2 to 6.0 for 25 per cent, pH 6.2 to 6.7 for 40 per cent, pH 6.2 to 7.2 for 55 per cent, and pH 6.9 to 7.3 for 70 per cent alcohol.

Comparisons of the results obtained under otherwise optimal conditions showed that there was a rapid increase in the adsorption and purification of the amylase with increasing concentrations of alcohol up to 25 per cent. At higher concentrations of alcohol, the adsorption of the amylase was somewhat less and the degree of purification, as measured by the yield of maltose per mg. of nitrogen, decreased. Thus in some typical experiments in which the optimal reaction was maintained in each case, the maximum adsorption of amylase expressed in terms of percentage of amylase present in the solution before the adsorption was about 15 per cent for 0 alcohol, 52 per cent for 10 per cent alcohol, 93 per cent for 25 per cent alcohol, 88 per cent for 40 per cent alcohol, 84 per cent for 55 per cent alcohol, and 80 per cent for 70 per cent alcohol. The maximum purification attained in each case expressed as mg. of maltose per mg. of nitrogen was about 23,000 for 10 per cent alcohol, 34,000 for 25 per cent alcohol, 32,000 for 40 per cent alcohol, 28,000 for 55 per cent alcohol, and 25,000 for 70 per cent alcohol. It was also noted that the total loss of active enzyme which was very low, about 2 to 5 per cent, in the presence of the 25 per cent alcohol was higher in the presence of the higher concentrations of alcohol.

The adsorption and purification of the amylase were found to increase with increasing concentrations of alumina, rapidly at first and then more slowly until almost complete adsorption of the amylase, about 90 to 95 per cent, occurred. Further increases in the concentration of the alumina or decreases in the concentration of the enzyme had no appreciable influence upon the purification as measured by amylase activity per mg. of nitrogen in the suspensions. In general, keeping the concentration of alumina as low as was consistent with good adsorption and purification resulted in better subsequent extraction and recovery of active amylase.

The results as a whole indicated that the maximum purification and recovery of the amylase occurred when the adsorption took place from solutions of about pH 5.2 to 6.0, containing 25 per cent alcohol (by volume) and with alumina gel equivalent to 0.60 to 0.80 gm. of $Al_2O_3$ for every 100 cc. of the enzyme solution.

*Extraction of Enzyme from Alumina Gel.*—Although the conditions now maintained during adsorption were different, our previous finding (1) that neutral or slightly alkaline solutions (pH 7.0 to 7.3 after adsorption) were most favorable for the extraction of this amylase from the alumina gel by which it had been adsorbed was confirmed.

The extraction of the amylase from the alumina gel by which it had been adsorbed and the activities of the subsequent precipitated preparations were also markedly influenced by other factors such as the nature and volume of the solvent, the time, the number of extractions, and the temperature.

Glycerol in various concentrations, with and without phosphate, 50 per cent alcohol, 0.01 M phosphate, and dilute solutions of sodium hydroxide were all investigated. When a relatively small volume of solution, about one-third to one-fourth of that of the solution from which the adsorption had occurred, was used, so that the dilution of the amylase was kept as small as possible and the reaction carefully controlled to keep the final hydrogen ion activity pH 7.0 to 7.3, almost as good extraction and recovery of the amylase could be obtained in the absence of glycerol and by the use of water made alkaline by means of dilute sodium hydroxide as in the presence of phosphate and glycerol. The extraction and the amylase activity of the solution in the absence of glycerol or phosphate were more variable than in the presence of either and more loss of active amylase often occurred during the extraction; but the elimination of phosphate and glycerol made unnecessary subsequent dialysis and resulted in products of the highest amylase activity when the solution was subjected to precipitation by alcohol and ether.

Willstätter and his coworkers (5) had advocated the use of 35 per cent glycerol in the extraction of the amylase from the alumina gel by which it had been adsorbed, but in our work, its presence at this point was found to be both unnecessary and disadvantageous. Even when low concentrations, 5 per cent or less, of glycerol were used, large losses of the amylase occurred during the dialysis before the glycerol was all removed, and traces of glycerol in the dialyzed solutions were found to interfere with the subsequent precipitation and drying of the enzyme.

The conditions resulting in the highest purification by the extraction of the amylase from the alumina gel also resulted in relatively large losses of active material. Attempts to recover more than 50 or 60 per cent of the amylase adsorbed were unsatisfactory. Prolonging the extraction of the alumina gel beyond that necessary for the thorough but rapid stirring and centrifuging of the ice-cold suspension, or the use of successive extractions with divided portions of a constant total volume, or with larger volumes, of a suitable solution, caused increased losses of active enzyme and also decreased the degree of purification by increasing the extraction of inert material as shown by the decrease in amylase activity per mg. of solid or of nitrogen.

The influence of all these factors is undoubtedly associated with the relatively rapid loss of activity of this amylase in solution which becomes greater as the degree of purification is increased. In general, the shorter the time of exposure, the less the dilution, and the lower the temperature, the better the results.

*Precipitation of Enzyme by Alcohol and Ether.*—The extracts obtained as above were precipitated directly without dialysis by adding the ice-cold clear solution to from one and one-half to twice its volume of ice-cold 1:1 alcohol-ether mixture.

*Properties of Dry Preparations.*—The average (and maximum) enzymic activity of thirteen preparations obtained as described above, from a commercial pancreatin[1] which had an activity (6) of 512 was 3760 (maximum, 4000), or the preparations were found to produce an average of 9500 (maximum, 10,000) times their weights of maltose from 2 per cent starch in 30 minutes at 40°.

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[1] We take pleasure in acknowledging our indebtedness to Parke, Davis and Company for this starting material.

These products are somewhat higher in activity than those obtained by our earlier method of fractional precipitation and dialysis, probably because the improvements of method here described minimize deterioration of activity during purification. We find no reason to doubt that the purified material is essentially the same as was obtained by our former method (2). It appears to be typically protein in nature, having a nitrogen content between 15 and 16 per cent in the dry ash-free substance and showing the precipitation and color reactions of typical proteins. The biuret, xanthoproteic, Millon, and ninhydrin reactions were all positive and precipitates were obtained when the solutions were treated with phosphotungstic acid, silver nitrate, or picric acid. Alcohol had a dehydrating effect similar to that observed with proteins and a preparation which had been treated with absolute alcohol lost its activity and was no longer appreciably soluble in water. Heating a solution of a purified preparation produced coagulation; the filtrate and coagulum both gave positive biuret reactions, the former a rose-violet and the latter a blue-violet color. They contained no detectable amounts of carbohydrate. Like our earlier preparations, these products also possess protein-splitting as well as starch-splitting activity. The difference in our results and those of Willstätter on this point has been discussed elsewhere (1).

Luers and Sellner (7) working with malt amylase, and Nishimura (8) working with the amylase of *Aspergillus oryzæ*, report that a second adsorption increased the purification of these enzymes; but when preparations obtained by the adsorption method described here or by our previous method of fractional precipitation by alcohol and ether and dialysis were dissolved, readsorbed, and otherwise treated as described here, it appeared that the optimal result had already been obtained in the manner described above. Contrary to the findings of Willstätter and his coworkers (5), the purified amylase was readily adsorbed by the alumina gel and the conditions which were optimal for the first adsorption of the relatively much less purified amylase were also optimal for the second adsorption of the purified enzyme. The conditions which had been found optimal for the subsequent extraction and precipitation of the amylase were also the best after the second adsorption.

Preliminary dialysis against alcohol, or against glycerol phosphate solutions, introduced before the adsorption by the alumina gel, when the amylase was not so highly purified and therefore not so rapidly inactivated, and carried out in the presence of glycerol to decrease the deterioration of the amylase in solution, caused no increase in the activity of the final products.

Washing the final precipitates with ice-cold 25 or 50 per cent alcohol, 50 per cent alcohol and ether mixtures, ether, or water, as well as attempts to redissolve and reprecipitate the enzyme, caused large losses of amylase activity. Grinding the dry preparations in a mortar also markedly decreased their activity. The properties of the purified material are being studied further.

### *Complete Method in Brief.*

1. Commercial pancreatin is extracted with 87 per cent glycerol, in the proportion of 10 gm. of pancreatin to 100 cc. of glycerol, by mechanical mixing for 6 hours at room temperature. The mixture is then centrifuged to remove the solid residue which is discarded.

2. Water-insoluble inert material is removed by addition of the extract to 5 times its volume of ice-cold water containing 0.012 M mono- and disodium phosphates in the proportions necessary to give a final reaction of pH 5.8. The exact proportions of the phosphates depend upon previous electrometric titrations of a similarly prepared enzyme solution. This diluted extract with a phosphate concentration of 0.01 M is centrifuged to remove the solids which are discarded.

Solutions obtained in (1) and (2) may be allowed to stand in the ice box for 12 to 24 hours without appreciable loss of amylase activity. All solutions and containers should be kept ice-cold. The centrifuge cups should be thoroughly chilled each time they are used. The room should be free from fumes and darkened.

3. 1 volume of the diluted extract from (2) is added to 2 volumes of an alcohol-alumina suspension made up as described in the "Note" below. The mixture is allowed to stand in ice with occasional shaking for not less than 15 minutes, when it is centrifuged and the liquid discarded. The final portion of "activated" alumina should be centrifuged and extracted as described in (4) below, within 30 minutes.

4. The "activated" alumina gel obtained in (3) is quickly but

thoroughly stirred in the centrifuge cup with ice-cold 0.015 M sodium hydroxide solution (to give pH 7.0 to 7.3) and immediately centrifuged. The volume of 0.015 M sodium hydroxide used for the extraction should be about one-third or one-fourth that of the enzyme solution treated with the alumina gel. In our experiments, 250 cc. centrifuge cups were used and the activated alumina gel in each corresponded to about 75 cc. of the diluted extract. Under these conditions, one extraction with 20 or 25 cc. of the faintly alkaline solution gave the best results.

5. The enzyme solution obtained in (4) is immediately filtered in the ice box and added to 1.5 to 2 times its volume of ice-cold 1:1 alcohol-ether solution. The precipitation is completed by the final addition of a few drops of ice-cold ether. The precipitate is immediately removed by centrifuge and dried on a watch-glass over sulfuric acid in a vacuum desiccator kept in the ice box. About 700 mg. of this highly active material may be obtained from 40 gm. of pancreatin.

*Note.*—An aqueous suspension of alumina gel free from sulfate is prepared in a manner similar to that described by Willstätter (9). Approximately 0.6 M aluminium sulfate is poured into an excess of 15 per cent ammonia solution. Steam is led through the alumina suspension thus formed for 10 to 12 hours to remove excess ammonia and increase the surface of the gel. The alumina gel is then washed by decantation until it no longer settles upon standing, when it is found to be practically free from sulfate. The $Al_2O_3$ content of aliquot samples of the suspension is then determined by evaporation to dryness and ignition to constant weight. The alcohol-alumina suspension used in (3) above is made up from the aqueous alumina suspension so as to contain alumina gel equivalent to 0.30 to 0.40 gm. of $Al_2O_3$ per 100 cc., 37 per cent alcohol, and 0.01 M phosphate. 2 volumes of this suspension when mixed with 1 volume of the enzyme solution as described in (3) above result in 0.60 to 0.80 gm. of $Al_2O_3$ per 100 cc. of diluted enzyme extract and a final alcohol concentration of 25 per cent in the enzyme-alumina gel mixture. The reaction of this solution after centrifuging to remove the alumina gel should be approximately pH 5.2 to 5.6. This should be verified electrometrically as deviations from this reaction markedly decrease the adsorption and purification attained. The proportions of the mono- and disodium phosphates required to give the above optimal reaction (pH 5.2 to 5.6) are best determined by previous electrometric measurements of a series of centrifuged solutions obtained after treatment of portions of an enzyme solution with a series of buffered alcohol-alumina suspensions which have been allowed to stand until equilibrium was reached with graded proportions of the acid and alkaline phosphates.

## SUMMARY.

A new method for the purification of pancreatic amylase has been developed. The method involves adsorption by alumina gel and subsequent precipitation by alcohol and ether and yields dry solid preparations.

The hydrogen ion activities of the solutions at the different steps in the process have a decided influence upon the results obtained and may be controlled and made reproducible by the presence of 0.01 M phosphate.

It was found feasible, in testing the reaction of the aqueous glycerol and alcohol solutions sometimes used, to make electrometric measurements with the hydrogen electrode and saturated calomel cell.

Slightly acid solutions, pH 5.2 to 6.0, favor the adsorption of the amylase by alumina gel, and slightly alkaline solutions, about pH 7.3, favor the extraction of the amylase from the alumina gel by which it has been adsorbed. This is in accord with the view that pancreatic amylase is of amphoteric nature.

It has been found possible to make use of glycerol in the early stages of the purification where it prevents or decreases the loss of activity of the amylase in solution and to omit its use in the later stages, after the adsorption, where it interferes with the precipitation of dry solid preparations. The active amylase may be effectively separated from glycerol by adsorption, but not by dialysis.

The yields of purified pancreatic amylase obtained by the method here described are larger, and these enzyme preparations are slightly more active, than those obtained by the Sherman-Schlesinger method.

Certain of the properties of the enzyme as here purified are described, and others are being studied further.

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# THE PROLAMINS OF DWARF YELLOW MILO AND FETERITA, TWO HORTICULTURAL VARIETIES OF HOLCUS SORGHUM.

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Of the three horticultural varieties of *Holcus sorghum,* kafir, milo, and feterita, the first was studied in this laboratory by Johns and Brewster (1). They were able to confirm von Bibra's (2) and also Osborne's (3) belief that kafir contained an alcohol-soluble protein. This new prolamin, termed kafirin by Johns and Brewster, was found to be so slightly soluble in cold 70 per cent alcohol that hot alcohol was used for its extraction from the meal. Hoffman and Gortner (4) prepared in a similar way a prolamin from the variety "early amber" which they named "*sorghumin.*"

We first examined milo and feterita seeds to ascertain whether they contained proteins of the globulin type. Inasmuch as Johns and Brewster made no statement in their publication regarding the presence of globulin in kafir, this variety of sorghum was also included in this investigation. It was found that 10 per cent sodium chloride solution extracted from each of the three sorghum meals a globulin which precipitated from the salt extract at 47 to 50 per cent of saturation with ammonium sulfate. The nitrogenous material extracted by the salt solution represented only 12.7 to 13.3 per cent of the total nitrogen in the meal of the three sorghum varieties, and not all of this was protein nitrogen. Because of the relatively small quantities present, no exhaustive study was made of the globulins.

Experiments were also conducted for the purpose of ascertaining whether these sorghum varieties contained proteins of the glutelin type. For this purpose the extractions were made with a 0.2 per cent solution of sodium hydroxide in 60 per cent alcohol rather than with hot alcohol, because Johns and Brewster showed that kafirin becomes denatured on boiling with 70 per cent alcohol. These alkaline-alcoholic extractions removed 73 per cent of the total nitrogen of feterita and kafir, whereas only 63 per cent of the total N of milo was similarly extracted. Acidification of the alkaline-alcoholic extracts caused no precipitation, indicating the absence of glutelins. After the alkaline-alcoholic extractions the residue was extracted with 0.2 per cent aqueous sodium hydroxide solution, but no further significant amounts of nitrogen were removed. Almost all the nitrogen in the alkaline-alcoholic extracts, therefore, represented prolamin nitrogen. These exhaustive extraction studies showed that prolamin is the predominant type of protein in the sorghum varieties mentioned above.

TABLE I.

*Elementary Composition of the Prolamins of Two Varieties of Holcus Sorghum in Percentages of Moisture- and Ash-Free Protein.*

| | Dwarf yellow milo. | Feterita. |
|---|---|---|
| Carbon | 55.25 | 55.11 |
| Hydrogen | 6.73 | 6.57 |
| Sulfur.* | 0.662 | 0.754 |
| Nitrogen | 14.95 | 16.30 |
| Ash | 1.313 | 0.576 |

* Sulfur was determined by the peroxide method by using Parr's heat ignition bomb (5).

## EXPERIMENTAL.

500 gm. of the meals prepared in the laboratory from milo and feterita seeds were extracted three times successively with 1 liter of 70 per cent ethyl alcohol at a temperature never higher than 60°. The combined alcoholic extracts were filtered rapidly into large shallow pans and then concentrated at room temperature by blowing air over them. After most of the alcohol had evaporated the aqueous liquid was decanted and the viscous precipitate which had separated and which represented the prolamin was redissolved in warm 70 per cent alcohol. After being filtered through paper pulp by suction, the prolamin was again precipitated from the clear filtrate by evaporation of the alcohol as described. The

product was finally dried with absolute alcohol and ether in the usual way. The yields based on the amount of meals taken were 3.3 per cent and 2.5 per cent of the feterita and milo prolamins, respectively.

TABLE II.

*Distribution of Nitrogen in the Prolamins of Four Varieties of Holcus Sorghum as Determined by the Van Slyke Method, Corrected for the Solubility of Bases.*

| | Dwarf yellow milo. | Feterita. | Kafir.* | Early amber.* |
|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* |
| Amide N | 20.61 | 20.63 | 20.76 | 18.96 |
| Humin " | 1.21 | 1.05 | 1.35 | 3.08 |
| Cystine N. | 1.20 | 0.82 | 1.23 | 0.92 |
| Arginine N. | 4.14 | 3.58 | 3.92 | 4.83 |
| Histidine N | 1.64 | 2.79 | 1.71 | 1.19 |
| Lysine N. | 2.89 | 2.18 | 2.48 | 3.45 |
| Amino N in filtrate from bases | 62.20 | 62.99 | 68.85 | 60.01 |
| Non-amino N in filtrate from bases | 5.68 | 4.54 | 0.32 | 5.33 |

* Analyses by Hoffman and Gortner (4).

TABLE III.

*Amino Acids of Dwarf Yellow Milo and Feterita Expressed in Percentages of Moisture- and Ash-Free Prolamins.*

| | Dwarf yellow milo. | Feterita. | Method of determination. |
|---|---|---|---|
| | *per cent* | *per cent* | |
| Arginine. | 1.92 | 1.86 | Calculated from Table II. |
| Histidine. | 0.91 | 1.68 | " " " II. |
| Lysine. | 2.25 | 1.85 | " " " II. |
| Cystine. | 0.60 | 0.64 | Sullivan (6). |
| Tryptophane. | None. | 1.29 | May-Rose (7). |
| Tyrosine. | 7.06 | 7.27 | Folin-Ciocalteu (8). |

The elementary composition of these proteins is shown in Table I. The distribution of nitrogen in the prolamins was determined by the Van Slyke method. The results, calculated on the basis of ash- and moisture-free proteins, are given in Table II. Although the elementary composition and distribution of nitrogen in the prolamins of milo and feterita reveal but little difference between these proteins, they are differentiated by their tryptophane content. The feterita prolamin was found to contain 1.29 per cent of tryptophane, whereas it is entirely lacking in the milo prolamin.

For the sake of comparison there are included in Table II the values for the prolamins of kafir and early amber as found by Hoffman and Gortner (4). Although these figures reveal no significant differences between these prolamins and those of feterita and milo, it is noteworthy that, like the milo prolamin, the prolamin of early amber was found to contain no tryptophane.

Tyrosine, tryptophane, and cystine were determined colorimetrically by the methods indicated in Table III. In connection with some tryptophane determinations carried on in this laboratory in 1925, it was found that the maximum color intensity was reached on the 5th day, both in the casein standard and in the proteins that were tested. The same length of time was also found to be required in the case of the feterita prolamin. The calculation was, therefore, based on the 5th day's reading. In the near future we shall publish in detail our experimental data in regard to this modification of the May and Rose method.

## SUMMARY.

Prolamin is the predominant type of protein in the seeds of the three horticultural varieties of *Holcus sorghum*, kafir, milo, and feterita. The prolamins of these seeds differ only slightly in their elementary composition and in the distribution of their nitrogen as determined by the Van Slyke method. The milo prolamin, however, is characterized by its lack of tryptophane. The feterita prolamin, on the other hand, was found to contain 1.29 per cent.

A globulin fraction precipitable from 10 per cent sodium chloride extracts of the meals at 47 to 50 per cent of saturation with ammonium sulfate was found in small quantities in each of the three varieties. The presence of glutelin could not be demonstrated.

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# UREA CONCENTRATIONS IN THE BLOOD OF THE RAT IN RELATION TO PREGNANCY AND LACTATION ON DIETS CONTAINING VARYING CONCENTRATIONS OF PROTEIN.*

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The determination of the blood urea is of considerable importance as an indicator of the metabolic activity of the organism and was of especial interest in the present investigation because of the possible significance attached to fluctuations in its concentration under the conditions of these experiments (Parsons, Smith, Moise, and Mendel, 1930) where both the excretory power of the kidney and a heightened protein metabolism were under scrutiny. The possible extent of its fluctuations in the blood under conditions of normally functioning kidneys in response to variations in the protein intake has been recognized (Addis and Watanabe, 1917; Wang, Hawks, and Wood, 1927).

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† Mary Pemberton Nourse Fellow of the American Association of University Women, 1927–28.

## *Experimental Procedure.*[1]

*Animals.*—The technique in regard to the care and handling of the rats has been fully described (Parsons, Smith, Moise, and Mendel, 1930). In addition to the rations already cited, two others were used: a high liver ration composed of beef liver 74.5 per cent, sodium chloride 1.0 per cent, calcium carbonate 1.5 per cent, butter fat 3.0 per cent, commercial dried brewery yeast 20.0 per cent, cod liver oil 1 cc. daily; and a modification of the Steenbock stock ration consisting of two-thirds by weight of the dry ingredients in that ration and one-third dried whole milk powder. This modification has also been employed by Waddell and Steenbock (1928) as an experimental ration.

*Methods of Analysis.*—Determinations of the urea concentration of the blood were made according to the micro urease method of Van Slyke (1927) by means of a portable manometric gas apparatus.

The blood samples for the determination of total solids were dried to constant weight in weighing flasks at 106°. Drying usually consumed 3 or 4 days time.

*Methods of Taking Blood Samples.*—Two methods were used in obtaining blood samples. For determinations both of urea and of total solids in the blood of the living animal samples were obtained from the tail, inasmuch as heart stabs were inadvisable in the case of the pregnant females. The perfect diffusion of urea makes the former method valid although it is less satisfactory for samples used for the determination of formed elements. One 0.2 cc. sample at a time was delivered into the cup of the Van Slyke apparatus except in the case of blood exhibiting a particularly rapid coagulation time. In such cases two 0.1 cc. samples were used. In a few instances a 0.2 cc. sample of blood was deposited in a small test-tube containing 1 cc. of 0.02 N lactic acid, was kept on ice, and later was transferred to the gasometric machine.

At the time the animal was sacrificed, it was anesthetized lightly and the skin dissected back from the ventral portion of the neck. An incision was made in the jugular vein and carotid artery

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[1] Thanks are due to Dora Hesse Goldschmidt and Eunice Kelly for technical assistance in the care of the experimental animals and in operative procedures.

on one or both sides with sharp pointed scissors. The blood was collected in a depression in a block of paraffin, care being used to hold the rat in such a position that the blood flowed over cut surfaces as little as possible, and drained directly from the elevated body of the rat. Samples were transferred to weighing bottles before clotting occurred.

*Time of Taking Blood Samples.*—The selection of the time intervals at which urea determinations were made depended on the possible significance of these intervals. In the first place a "base-line" was sought against which to measure possible fluctuations occurring in other periods. For this purpose determinations of the urea concentration were made on the blood of the rats while they were still on the modified Sherman stock ration immediately before they were nephrectomized and changed to the new experimental conditions and diet. It would have been desirable to include another control determination at a given interval after operation and the change in diet, but before gestation. However, the possibility of breeding some of the animals in so short a time as a week after the operation made this period impractical.

The first experimental period selected for determining the concentration of urea in the blood was as early in the first 2 weeks of gestation as convenient. A second period was one shortly before parturition. Inasmuch as the beginning of gestation had been determined by the vaginal smear, and the length of gestation usually was 22 or 23 days although occasionally extending to even 25 days, the time of its probable termination could be foretold with relative accuracy. This period is known to be a time of rapidly forming tissue in the fetus. Two periods during lactation, namely near the 14th and the 21st days, were also chosen for urea determinations in the case of most of the females with litters, with a view to including the time of greatest need for food on the part of the mother rat and therefore presumably the height of protein metabolism. Samples were obtained in a few instances following weaning.

The opportunity to determine the urea concentration in the blood of both intact and partially nephrectomized rats afforded a possible criterion of the adequacy of the physiological adjustments to the conditions of the experiment. A greater concentration of urea in the blood of the latter animals might reasonably be interpreted as the degree of failure to compensate on the part of the remaining kidney.

The number of determinations performed rendered uniformity in the time of day at which these were done practically impossible. The suggestion arose of subjecting the rats to a preliminary fast before the blood sample was drawn. Since the time of day of sampling varied, a fast, unless it lasted from 14 to 22 hours, would give only an appearance of accomplishing uniformity without actually doing so. This is because the distribution of the food intake by the rat is so uneven during the 24 hours, two-thirds or more of the total food ingested being consumed at night. Moreover, a period of fasting would have defeated one purpose of the experiment; namely, to determine the actual degree of protein

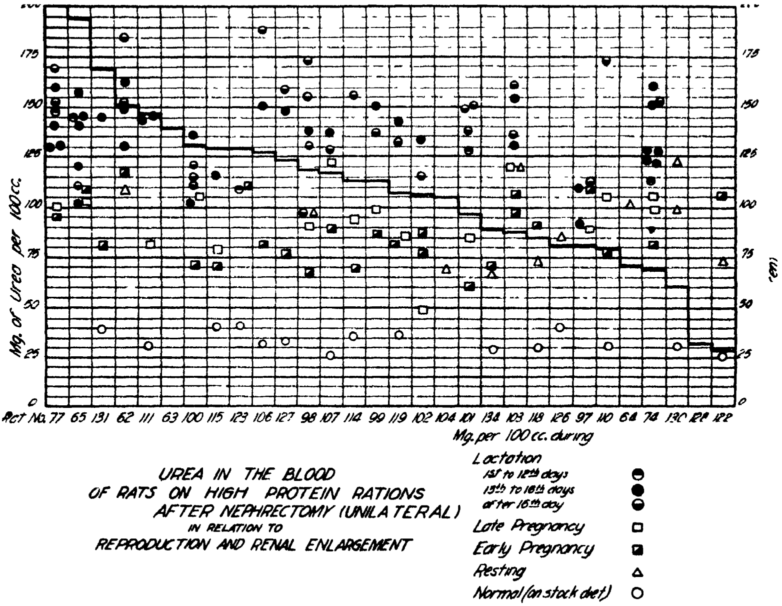


FIG. 1. The dots indicate the concentration of urea in the blood of rats before and after the removal of one kidney and the feeding of an experimental diet at various periods with reference to reproduction. The heavy line indicates the percentage of enlargement of the remaining kidney based on the heart weights of these rats.

mobilization in these animals under the conditions imposed in so far as this was reflected in the concentration of urea in the blood.

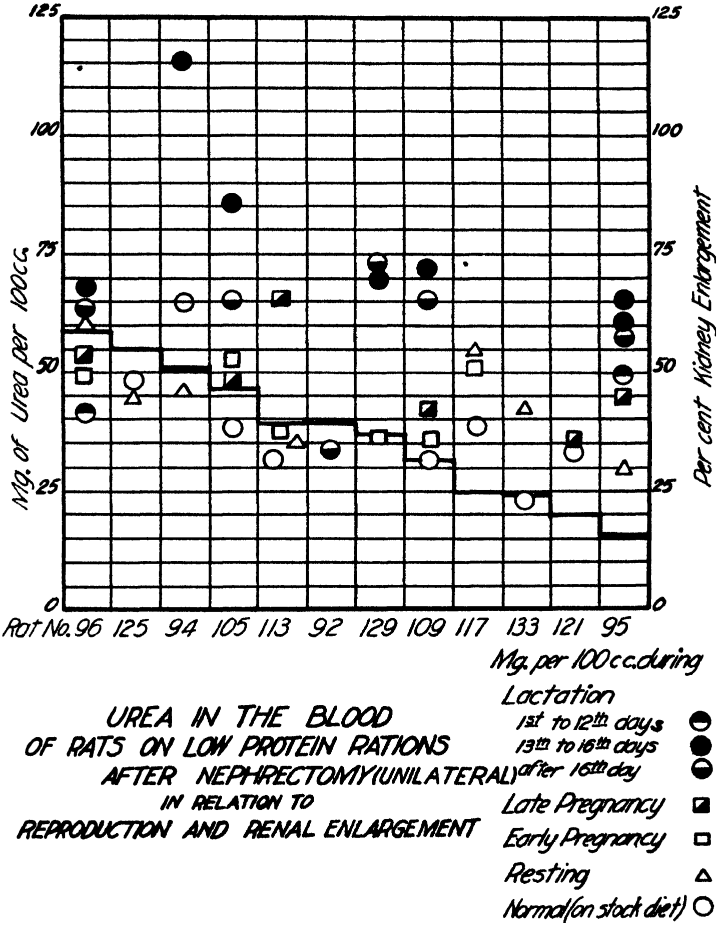


FIG. 2. The significance of the symbols and heavy line is the same as for Fig. 1.

## *Results.*

*Variations in Urea of Blood at Different Periods.*—The "normal" values in this experiment for the concentration of urea in the blood of rats on a stock ration immediately before nephrectomy ranged from 23 to 48 mg. per 100 cc. of blood, values roughly comparable to those obtained in the Yale laboratory for male rats under similar conditions. In striking contrast to these low figures, however, were the wide fluctuations in the urea content of the blood of these same animals when subjected to the experimental conditions. Figs. 1 to 3 present the results of an extensive series of urea determinations in the blood of partially nephrectomized and intact rats with both high and low protein intake.

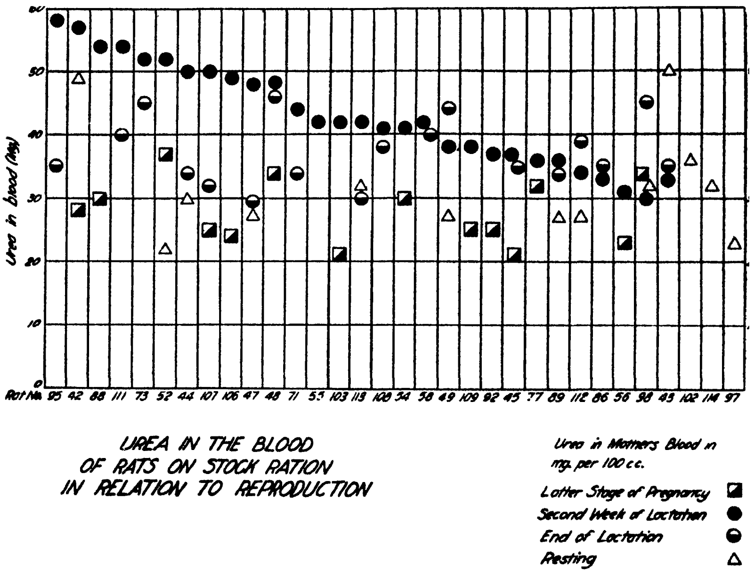


FIG. 3.

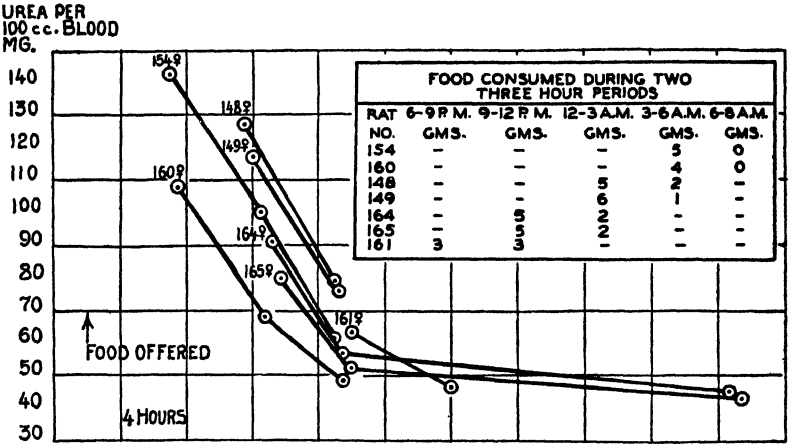
By comparing the records of the rats on high protein rations with those on low protein it can be seen that dietary protein exerts a profound influence on the blood picture in regard to urea content. Similarly, a comparison of the figures for animals on any of the diets during lactation as compared with intervals of rest shows that periods of milk production are associated with an increased concentration of urea in the blood. The results are inconclusive in regard to the influence of gestation alone, since this was not differentiated clearly, and there is no reason to interpret the data as showing any influence of this factor. Certainly the influence of lactation is of so much greater magnitude in comparison that the former is relatively insigificant. A comparison of the intact with the partially nephrectomized animals on high protein rations showed that both groups respond to this dietary influence with an increase in concentration of urea in the blood but that the latter group did so to a much greater degree. The results would seem to indicate that a single remaining kidney after nephrectomy, even though hypertrophied, is not the equivalent in function to the two normally present.

*Testing Various Hypotheses to Account for Wide Variations Observed.*—While considerable information had thus been compiled concerning concentrations of urea in the blood of rats under varying conditions of diet, reduction of kidney tissue, and stages of reproduction, it was highly desirable to arrive at a better understanding of the mechanism regulating such striking fluctuations as had been observed.

A careful examination of the data gave assurance that the time of day of taking samples was not in itself responsible for the highest and lowest values recorded, although this possibility might be suggested in view of the predominately heavy night feeding of the rat. Even when samples had been taken at more than one time of day from the same lactating females no clear tendency was observed for the peak to fall at any one time although great differences (as high as 30 mg.) were noted between concentrations of urea in the blood of the same rat at different times of day.

A detailed record was next kept of the daily food intake of a group of twenty nephrectomized rats, some pregnant and some lactating. On the days the urea determinations were made, the rats fasted from 9 a.m. until the blood sample was drawn. For intakes of 17 to 14 gm. of food the blood urea concentrations were uniformly high, 132 to 104 mg. When 1 gm. or less of food was eaten, the urea values were uniformly low, 46 to 25 mg., but between these extremes, urea values from 127 to 48 mg. seemed to be scattered impartially without reference to previous food intake or time of day of taking the sample. In the case of three samples taken at 5.30 p.m. for example, the urea concentration of one was 81 mg. with a food intake of 13 gm., 105 mg. for another with the same food intake, and 104 mg. for the third with an intake of only 3 gm. of food. Clearly, some other factor besides the total consumption of food was causing the variations.

Two other hypotheses were entertained. It might be that the consumption of food by some rats was concentrated in a short space of time either early or late in the night interval. On the other hand it was possible that the excretory capacity of the kidney differed markedly from rat to rat, resulting in varying degrees of retention of urea. Accordingly an experiment was planned to test the validity of these general hypotheses by controlling more carefully the exact time of consumption of a given amount of food. 7 gm. of food were offered to each of seven rats and the uneaten portions were ascertained at the end of a 3 hour interval and again at the end of a second 3 hour interval, at which time any uneaten portion was withdrawn and fasting continued throughout the remainder of the night and the following day. The 6 hours allowed for feeding began for different rats at 6 p.m., 9 p.m., 12 p.m., and 3 a.m., respectively. The curves given in Fig. 4 were plotted from the two or more urea determinations made at intervals during the following day for each animal but they are arranged on the sheet not relative to the actual hour of the taking of the sample but with reference to the interval elapsing after food was offered to the animals at some given time in the night.



FOOD CONSUMED DURING TWO THREE HOUR PERIODS

| RAT NO. | 6-9 P.M. GMS. | 9-12 P.M. GMS. | 12-3 A.M. GMS. | 3-6 A.M. GMS. | 6-8 A.M. GMS. |
|---|---|---|---|---|---|
| 154 | – | – | – | 5 | 0 |
| 160 | – | – | – | 4 | 0 |
| 148 | – | – | 5 | 2 | – |
| 149 | – | – | 6 | 1 | – |
| 164 | – | 5 | 2 | – | – |
| 165 | – | 5 | 2 | – | – |
| 161 | 3 | 3 | – | – | – |

**Fig. 4. Decline in the concentration of urea in the blood of rats following definite intervals of feeding.**

The curves in Fig. 4 showing the decline in the urea content of the blood at given intervals after the ingestion of food are remarkably uniform. There would seem to be no indication of any retention of urea in these seven rats. The rapidity of the decrease in the concentration of urea and the relatively low values reached in the blood of these rats after the withdrawal of food indicate that the functional capacity of their surviving renal tissue after the removal of one kidney is essentially unimpaired, after certain intervals on a high protein diet.

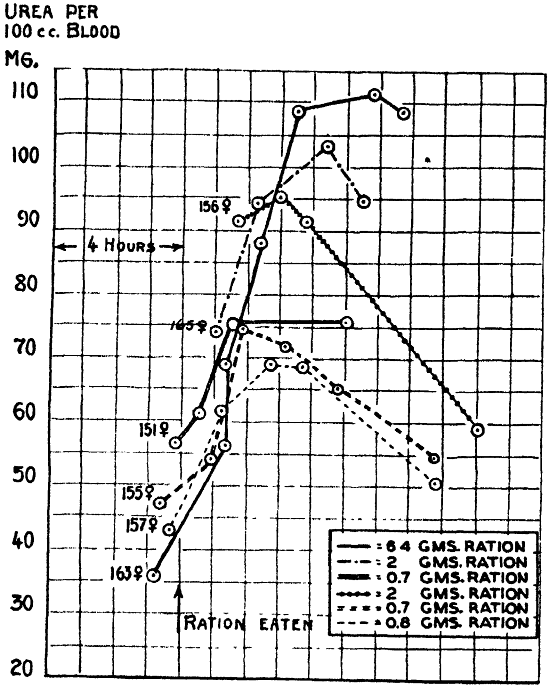


Fig. 5. Changes in the urea concentrations of the blood of rats soon after feeding, showing the initial increases to be roughly proportional to the intake of food.

On the other hand there seemed to be some possibility that distribution of food intake in point of time might account for the apparent discrepancies which had been encountered previously in attempting to correlate total food intake with urea content of the blood. If the curves in Fig. 4 are arranged, not with reference to time after feeding but with reference to the time of taking the blood samples, it is evident that, at a certain hour of the day, the content of urea in the blood of Rat 154 was 105 mg. per 100 cc. of blood, and for Rat 161, 53 mg. (interpolations on the curves), although the amount of food eaten was 5 gm. in the one case and 6 gm. in the other.

Since such uniform results were obtained for the rapid decrease in concentration of urea during an interval after feeding, it was of great interest to determine and plot the rise as well as the fall of urea in the blood after a definite amount of food intake. Two rats were deprived of food during the night time and in order to reverse as far as possible diurnal conditions, the rats were kept under a strong electric light during the night and in the morning were placed in a dark room with a weighed amount of food. Within half an hour approximately 2 gm. of food had been consumed by each rat after which time the rats appeared sleepy and not inclined to eat more. Food was, therefore, withdrawn and urea determinations were begun immediately. The graphs (Rats 156 and 165, Fig. 5) show the results of four successive urea determinations for each animal.

Since even these prompt determinations had apparently not caught the initial part of the response to the ingestion of food, the ration was withheld from four other rats during the night, and the following morning before any food was offered determinations were made of the fasting level of urea in the blood (Fig. 5). These fasting values were found to be comparatively low. After taking the blood sample, food was offered to the rats and the time when they actually began eating was recorded as the beginning of the food intake period. 0.7 gm. of ration was consumed by each of two rats, Nos. 151 and 155, 0.8 gm. by Rat 157, and 6.4 gm. by Rat 163, the latter eating this amount in 17 minutes.

It is apparent from Fig. 5 that even in the short interval of half an hour after the beginning of food consumption a distinct increase in the concentration of urea in the blood had occurred. This would seem to indicate an amazing speed of digestion, absorption, and deaminization. The curves also show a surprizing uniformity. The intake of even relatively small portions of a high protein ration after a preliminary fast and the drawing of a control blood sample is thus shown to be followed by a rapid increase in the concentration of urea in the blood of partially nephrectomized rats, from a relatively low fasting value to a concentration roughly proportional to the amount of food consumed. The concentration subsequently decreases but at a somewhat slower rate than the increase.

TABLE I.

*Relationship of Total Solids to Content of Urea in Blood of Rats Fed Different Concentrations of Protein in Ration.*

| Rat No. | Identity.* | Sample for total solids. | Date of sample for total solids | Amount of total solids. | Urea. | Date of sample for urea. |
|---|---|---|---|---|---|---|
| | | | | *per cent* | *mg. per 100 cc.* | |
| 106 | NH | Tail. | Apr. 6 | 21.6 | 188 | Simultaneously. |
| 62 | " | " | " 4 | 21.7 | 182 | " |
| 77 | " | Carotid. | " 1 | 20.9 | 170 | Mar. 30. |
| 101 | " | " | Mar. 28 | 21.1 | 151 | " 27. |
| 103 | " | Tail. | Apr. 6 | 21.9 | 136 | Simultaneously. |
| 119 | " | " | Mar. 29 | 22.1 | 133 | " |
| 74 | " | " | " 29 | 22.1 | 129 | " |
| 74 | " | Carotid. | " 30 | 20.9 | 129 | Mar. 29. |
| 102 | " | " | " 29 | 21.3 | 115 | " 27. |
| 65 | " | Tail. | Apr. 10 | 23.4 | 112 | Simultaneously. |
| 126 | " | " | Mar. 29 | 21.0 | 87 | " |
| 122 | " | Carotid. | " 31 | 21.4 | 75 | Mar. 31. |
| 134 | " | " | " 28 | 20.7 | 68 | " 27. |
| 124 | IH | Tail. | Apr. 10 | 21.5 | 67 | Simultaneously. |
| 137 | " | Carotid. | " 3 | 20.3 | 63 | Mar. 31. |
| 136 | " | " | " 3 | 20.7 | 54 | " 31. |
| 129 | NL | Tail. | Apr. 10 | 20.4 | 74 | Simultaneously. |
| 117 | " | " | " 10 | 21.2 | 71 | " |
| 125 | " | Carotid. | " 6 | 20.7 | 47 | Apr. 5. |
| 133 | " | Tail. | Mar. 29 | 21.1 | 43 | Simultaneously. |

* The identity is indicated by the symbols as follows: NH, rat nephrectomized and fed high protein diet; IH, rat intact and fed high protein diet; NL, rat nephrectomized and fed low protein diet.

Although the experiments just described were suggestive, they seemed inadequate to explain fully the very high urea concentrations of 160 to 180 mg. which had been observed in the blood of some of the lactating rats. It is conceivable that in the lactating rats fed the high protein rations there would be a relative anhydremia, not only due to the large mobilization of fluid necessary for excreting metabolites in the urine but also to the elimination of water in the milk secreted. Accordingly determinations were made of the total solids in the blood of certain of the rats as recorded in Table I.

From the third and fifth columns in Table I it is plain that there is no direct correlation between the concentration of urea and of total solids in the blood of these animals. Variations in the water content of the blood are therefore not an explanation for the very wide fluctuations in the concentrations of urea observed in the blood of nephrectomized rats fed diets rich in protein.

It had been noted that the lactating mother rats whose daily food intake reached as high as 19 or more gm. consumed an appreciable amount of food during the daytime. It seemed possible that the extremely high urea values of 160 to 180 mg. per 100 cc. of blood which had been observed previously might have resulted from a summation of metabolites from small portions of food consumed throughout the day superimposed on the heightened urea concentration due to the large intake of food during the night. Accordingly observations were made on two lactating females. The first determinations of urea showed approximately the same concentrations in the blood of each. The food cup was allowed to remain in the cage of Rat 153, and a sharp increase in the concentration of urea in the blood of this animal was observed at the end of an hour's time. Food was withdrawn from Rat 151 and the urea concentration was found to have fallen considerably at the end of a 5 hour interval. At the outset, therefore, these results seemed to be in harmony with the hypothesis that the excessively high concentration of urea which had been observed in the blood of lactating rats could be adequately explained in the basis of the effect of small portions of food eaten frequently during the day, after heavy night feeding; however, later results made a different interpretation of this experiment necessary. Simultaneous observations were next made on Rats 150 and 153, both of which were suckling litters. Food was withdrawn and a comparable decline in the urea concentration was observed to take place in both. Following this preliminary determination of the trend of the curve, food was offered to Rat 150 but was still withheld from Rat 153. Quite contrary to expectation, the next determinations showed a sharp increase in the concentration of urea in the blood of Rat 153

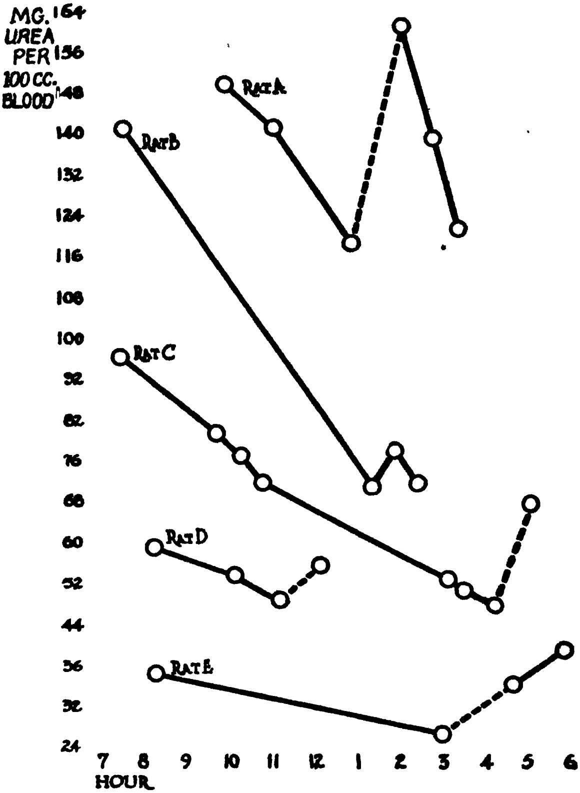


Fig. 6. Changes in the concentration of urea in the blood of rats with reference to suckling their young. The solid lines denote values for mother rats without food or young. The dotted lines denote that the young were allowed to suckle for part of the time. Rats A, B, C, and D had one kidney; Rat E, two kidneys. Rats A and B were fed a high casein ration; Rat C was fed a high liver ration; Rats D and E were on stock rations.

from which food had been withheld and a continued decline in the blood of Rat 150 which had eaten food.

Rat 153 had been suckling a litter at the time that the blood sample was taken for the third determination so that the young had to be literally shaken free from the mother's nipples. It seemed barely possible that the act of nursing might itself have some influence although the results of previous determinations of blood solids gave no support to the hypothesis that unusual concentrations of the blood of the mother was the factor responsible. Therefore, a series of determinations of blood urea was made on all of the individual rats of the group with litters. Food was withdrawn in the morning at as early an hour as possible and the mother rat segregated from her young. Urea determinations were made immediately and at succeeding intervals during the day. When a considerable decline in the concentration of urea in the blood had been observed in each rat, the litter of young was restored to the mother's cage without, however, the food cups being replaced. The graph for Rat A on Fig. 6 is representative of the response obtained from each of the six mother rats in this group.

It is seen that following the decline in the urea content of the mother's blood, a sharp increase occurs after the young have suckled. The rise was observed at even as short an interval as 20 minutes after the beginning of suckling. The conclusion seems warranted that the puzzling observation made earlier, of the rise in concentration of urea in the blood of Rat 153 without food was due to the active nursing of the young. It is also probable that the same explanation accounts for the increase first observed for this same rat, thought at that time to be due solely to an intake of food.

Although the results demonstrated that prompt and striking increases in the concentration of urea in the blood of partially nephrectomized rats on a high casein ration may be produced in response to the act of withdrawal of milk by the young even though food has not been consumed by the mother rat for several hours previously, it was not clear whether the phenomenon was general in nature or occurred only under the special conditions of high casein feeding and partial nephrectomy. Accordingly, further experiments were carried out on lactating rats fed rations rich in casein, liver, or egg albumin, as well as rations relatively low in protein. Both intact rats and those in which one kidney had been removed were fed stock rations.

The physiological response was found to be decidedly general inasmuch as distinct increases in the urea content of the blood were observed in each type listed above. Of the 55 periods of suckling studied, forty-six showed an augmented concentration of urea in the blood of the mother following a half hour to an hour of nursing. Typical graphs of the individual experiments are shown in Fig. 6.

The type of protein fed seemed to exert some influence. The average concentration of urea in the blood of the mother rat at an early hour of the day when the experiments were begun was greater in those cases where high casein or high egg albumin was fed than with a ration containing an approximately equal concentration of nitrogen from liver. Furthermore, the magnitude of the average increase following nursing was greatest after previous feeding of casein-rich rations. These relationships are made clear in Table II.

TABLE II.

*Concentration of Urea in Blood of Lactating Rats as Related to Diet and Reduction of Renal Tissue.*

| | Rats with one kidney. | | | | Rats with two kidneys. |
|---|---|---|---|---|---|
| Type ration fed | High casein. | High liver. | High egg albumin. | Stock. | Stock. |
| No. of periods of nursing studied | 17 | 18 | 13 | 4 | 3 |
| Average increase in concentration of urea in blood of mother rat following nursing period, *mg. per 100 cc. blood* | 18.6 | 6.5 | 15.1 | 12.0 | 6.0 |
| Greatest single increase in urea in blood of mother following nursing period, *mg. per 100 cc. blood* | 50.0 | 31.0 | 29.0 | 19.0 | 10.0 |
| No. of nursing periods not followed by increase in urea content of blood | 2 | 7 | 0 | 0 | 0 |
| Average concentration of urea in blood at beginning of experimental day, *mg. per 100 cc. blood* | 138.8 | 107.3 | 123.3 | 81.0 | 39.0 |
| Highest single determination of urea during day, *mg. per 100 cc. blood* | 191.0 | 150.0 | 159.0 | 117.0 | 67.0 |

An important point to be determined because of its bearing on the nature of the phenomenon observed was whether or not the degree of increase in urea concentration in the blood is proportional to the amount of milk obtained by the young in nursing. While this question could not be settled with certainty by any of the methods that suggested themselves, significant data bearing on this point were obtained by weighing the young just before and just after nursing. The amount of milk estimated by this method showed so imperfect a correlation with the apparent vigor of the efforts of the young to nurse that later, in thirty-two instances, the weights of the mother rats were recorded also. In twenty-five of these, the increase in weight of the litter failed to equal the loss in weight of the mother by from 3 to 7 gm., for some undetermined reason. A loss of weight of the young due to excreta does not seem to be the factor involved. However, in seven of the thirty-two cases, these discrepancies were not found.

TABLE III.

*Fluctuations in Urea Content of Blood of Mother Rats in Relation to Amount of Milk Nursed by Young a Short Time Previously.*

| Increase in concentration of blood urea per 100 cc. between samples before and after nursing period. | Loss in weight of mother rat. | Gain or loss in weight of whole litter. |
|---|---|---|
| *mg.* | *gm.* | *gm.* |
| 19.5 | 0.0 | −4.0 |
| 15.0 | 0.0 | −4.0 |
| 20.0 | 0.0 | −2.0 |
| 8.4 | −3.0 | +2.0 |
| 3.2 | −4.0 | +3.0 |
| 10.4 | −8.0 | +6.0 |
| 6.3 | −13.0 | +12.0 |

Three cases were selected where a lack of decrease in weight on the part of the mother gave assurance that no significant amount of milk was obtained by the young, for a group on the one hand; four others were selected where the loss of weight on the part of the mother was practically equalled by the gain in weight on the part of the young, thus lending validity to the figures as a true estimate of the amounts of milk obtained, on the other hand. A comparison of the increases in the concentration of urea in the blood of the mother rats in the two groups would then be a crucial test of the question raised above as to whether these increases in urea are proportional to the amount of milk nursed.

From the data in Table III it seems obvious that the increases observed in the urea content of the blood of lactating rats are not proportional to the amount of milk nursed. While the figures for urea cannot be taken to represent exactly the total increases for each period, inasmuch as the maximum height of the increase may not have been revealed by the sample obtained, nevertheless the fact that increases in urea content occurred at all when evidently no milk was obtained shows that the increases in urea and the amount of milk nursed are not correlated. This observation is of importance inasmuch as the first hypothesis assumed was that the source of the urea causing the increased concentration might be incident to a rapid formation of milk during or following nursing to replace that withdrawn by the young. This hypothesis therefore cannot be correct. It is, however, not inconceivable that the sensation of pulling at the teats might normally initiate the mobilization of protein for milk formation through some reflex mechanism inasmuch as this sensation would usually be accompanied by the withdrawal of milk from the mammæ.

The discovery that the fluctuations in urea values in the blood are not proportional to the amount of milk withdrawn suggested, however, the possibility that the whole phenomenon might be a response of the animal to the emotional excitement incident merely to obtaining the blood samples. This possibility had seemed at first to have been obviated by the taking of two or more blood samples before nursing, following the withdrawal of food, and finding so pronounced rates of decline in the urea concentration in the blood in this preliminary period. The question had not been entirely answered, however, inasmuch as the intervals between the preliminary samples were usually not so short as the intervals between the samples taken before and after nursing respectively and hence the possibility still remained that a rise in urea content had followed the taking of the former samples, but had been of so brief duration as to have escaped detection. The reason that the two or more preliminary blood samples were not taken more closely together in the experiments from the first was that it was thought important to disturb the rat as little as possible just before nursing in order to avoid excitement and induce an adequate flow of milk. However, in certain of the experiments this consideration was set aside and blood samples were taken at brief intervals without nursing. In only thirteen of the 95 intervals without nursing where the trend of the urea content was determined was there any increase found. Of these, eight instances occurred at an early hour in the day following the removal of the ration cup and may have been due entirely to the metabolism of protein recently eaten. In five cases, however, illustrated by rat B on Fig. 6, increases seem really attributable to the disturbance incident to the blood sampling, and may be a confirmation of Tashiro's results. He has shown (1925, 1926) that the urea nitrogen concentration of the blood of rabbits is increased by parasympathetic stimulants such as pilocarpine and choline, or by directly stimulating the peripheral stump of the vagus but is decreased by injections of atropine and adrenalin or by the direct stimulation of the splanchnics or the central stump of the vagus. He noted also a prompt increase in the urea concentration of the blood of rabbits during the process of binding them. After 3 to 5 hours of binding, the urea concentration was sometimes found to be doubled. He attributed this phenomenon to the central stimulation of the vagus. Astanin and Rubel (1928) confirmed the results obtained by Tashiro by stimulation of the peripheral end of the vagus in contrast to the failure to increase the urea concentration of the blood on stimulating the sympathetic nerves. They suggested the possible nervous influence on some gland of internal secretion.

The results of the experiments with rats in the present instances, however, clearly demonstrate that the increases in urea in the blood following nursing are of unmistakably greater magnitude than those resulting from the blood sampling itself. This is illustrated in Fig. 6 by the graph of Rat C, where repeated samples of blood taken at close intervals during the day showed a steady decrease in the urea content of the blood; but on the other hand, the sample taken following a nursing period late in the day showed the typical response of augmented urea.

## DISCUSSION.

In view of the multiplicity of the influences which have been shown in this investigation to have a bearing on the urea concentration in the blood, and the wide fluctuations occurring during even an hour's time, there seems no justification for an attempt to formulate an exact mathematical expression of the values for urea in the blood of these experimental animals, such as has been devised by MacKay (1928) to express the relationship between the renal weight of her experimental rats and the concentration of urea in their blood derived from food protein, based on single determinations.

On the other hand these experiments are suggestive of such possible relationships in a qualitative way. The larger relative size of the kidneys of the young of mother rats on high protein rations (Parsons, Smith, Moise, and Mendel, 1930) as well as the very striking enlargements of the kidneys of these females themselves in comparison with non-lactating rats, seems to be correlated with blood changes. One might postulate an influence due to the concentration of nitrogenous metabolites circulating in the mother's blood and perhaps passing into her milk in more than usual amounts. But although the fluctuations in the blood urea were the observed phenomena associated with nursing, it would be an unwarranted assumption to restrict the possible agent in the renal changes in mother and offspring to urea itself, inasmuch as the concentrations of other metabolites were not determined simultaneously. It is conceivable that some substances more physiologically active than the relatively inert urea are also involved in the mobilization of nitrogen of which the urea fluctuations in these experiments seem to be an index.

## SUMMARY.

Striking fluctuations were observed in the urea concentration in the blood of rats subjected to varying conditions in respect to reduction of kidney tissue, concentration of dietary protein, and the burdens of reproduction.

The highest concentration of urea, 191 mg. per 100 cc. of blood, occurred in a lactating rat with one kidney, on a high casein ration.

It has been shown that the increased food intake of the lactating females accounts only in part for the extreme values observed. A definite influence of the act of suckling itself on the concentration of urea during fasting has been demonstrated in the blood of intact rats as well as those with only one kidney, when the ration consumed previous to the fast was rich in casein, liver, or egg albumin, or contained only moderate amounts of protein.

Inasmuch as the increases in the concentration of urea in the blood of the lactating rat following the suckling of the young seem not to be closely correlated with the amount of milk nursed, it is possible that they result from an emotional response to the act of suckling itself rather than from the formation of milk in the mammary gland.

An occasional slight increase in the urea concentration occurred following the taking of the blood sample without nursing, and may be related to the effect on blood urea shown by others to result from manipulation of the experimental animal. However, the increases in urea in the blood of rats following nursing were demonstrated to be of unmistakably greater magnitude than those resulting from the blood sampling itself.

It is a pleasure to acknowledge the generous assistance of Professor Arthur H. Smith and Professor Lafayette B. Mendel of the Laboratory of Physiological Chemistry and Dr. T. S. Moise of the Department of Surgery of Yale University both in guiding the research during its early phase and in revising the manuscript.

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# THE EFFECT OF PHOSPHORUS ADMINISTRATION, ANTI-RACHITIC TREATMENT, AND SPONTANEOUS HEALING ON THE CALCIUM IN THE SERUM OF RACHITIC RABBITS.

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It has been shown that the administration of phosphorus in large doses may lead to a decrease of the serum calcium (1–6), although, in the experiments published, the decrease of the calcium does not show any regular quantitative relationship to the simultaneous increase in the serum phosphorus. It is possible that the phenomenon indicates saturation or supersaturation, although it must be kept in mind that the conditions in the serum are not directly comparable to those in an inorganic solution in contact with the solid phase.

The failure of the bones to calcify in rickets and the low values for phosphorus found in the serum in this disease, would lead one to believe that the serum in rickets would not show this phenomenon, whatever its significance may be. In the work published by Orr, Holt, Wilkins, and Boone (6), however, it was shown that phosphorus administration to a rachitic infant may also lead to a decrease of the serum calcium. It seemed to us that this was an important observation and we have, therefore, administered phosphorus to rachitic rabbits and determined the serum calcium and phosphorus before and after the administration. The results may be seen in Table I. The administration of phosphorus has in every case, except one, led to a definite decrease in the calcium concentration. In one case the phosphorus concentration increased to a value of normal magnitude, in another it increased to a value higher than normal. In the remaining cases the phosphorus after the administration, although higher than before, was still lower than normal, but the decrease of the calcium has taken place also in these cases.

In another series of experiments we have raised the concentration of phosphorus in the serum of rachitic rabbits by the administration of cod liver oil, irradiated ergosterol, or ultra-violet light (Table II). Only those cases are included in the table where the treatment resulted in an increase of the phosphorus of at least 0.25 millimols per liter of serum. Also when the phosphorus was increased by these therapeutic agencies, a decrease of the calcium generally occurred. It is obvious that the effect must be one of only temporary duration. (We have also a series of animals given rachitic diet and cod liver oil for longer periods, and these animals show, as might be expected, perfectly normal values both for calcium and phosphorus; this is such a well known finding that we do not think it worth while to include these data in our paper.) The animals in Table II who received cod liver oil for 3 or 4 days all show a decrease of the serum calcium. The animals having received cod liver oil for 5 days do not all react in the same way; one shows a very marked decrease of the calcium, in two cases the values are about the same before and after treatment, and in one the calcium is higher after the administration of cod liver oil. When ergosterol was given, five animals showed (after 2 days) a

TABLE I

*Effect of Phosphorus Administration (Secondary Sodium Phosphate) on the Serum Calcium in Rachitic Rabbits.*

Values are expressed in millimols per 1000 cc. of serum.

| Experiment No. | P | | Ca | | Remarks |
|---|---|---|---|---|---|
| | Before P administration. | After P administration. | Before P administration. | After P administration. | |
| 8 | 0.87 | 1.52 | 3.47 | 2.93 | 10 mg. P intraperitoneally. |
| 13 | 0.87 | 1.08 | 3.48 | 2.65 | 5 “ “ “ |
| 148 | 0.97 | 1.59 | 3.35 | 2.63 | 500 “ “ by mouth. |
| 158 | 0.65 | 0.87 | 3.10 | 2 85 | 159 “ “ “ “ |
| 168 | 0.40 | 0.63 | 3.07 | 2 83 | 159 “ “ “ “ |
| 201 | 1.06 | 4 61 | 3.10 | 1.44 | 79 “ “ intraperitoneally. |
| 207 | 0.87 | 2.65 | 3 23 | 3.18 | 79 “ “ “ |

TABLE II.

*Effect of Cod Liver Oil, Light, or Irradiated Ergosterol on the Serum Calcium.*

Values are expressed in millimols per 1000 cc. of serum.

| Experiment No. | P | | Ca | | Remarks. |
|---|---|---|---|---|---|
| | Before treatment. | After treatment. | Before treatment. | After treatment. | |
| 217 | 0.86 | 1.76 | 2.50 | 2.15 | 1 cc. cod liver oil daily for 3 days. |
| 224 | 0.79 | 1.20 | 3.15 | 2 41 | 1 " " " " " " 3 " |
| 226 | 0.76 | 1.07 | 2.75 | 2.66 | 1 " " " " " " 4 " |
| 231 | 0.58 | 1.82 | 3.07 | 2.40 | 1 " " " " " " 4 " |
| 232 | 0.88 | 1.62 | 3.00 | 2.78 | 1 " " " " " " 4 " |
| 235 | 0.81 | 1.19 | 3.34 | 3.18 | 1 " " " " " " 4 " |
| 225 | 0.70 | 0.95 | 3.08 | 3.40 | 1 " " " " " " 5 " |
| 227 | 0.60 | 1.30 | 3.10 | 3.08 | 1 " " " " " " 5 " |
| 229 | 0.68 | 1.06 | 3 10 | 3.14 | 1 " " " " " " 5 " |
| 234 | 0.97 | 3.00 | 3.23 | 2.04 | 1 " " " " " " 5 " |
| 237 | 0.27 | 1.36 | 3.03 | 2.89 | 1 " ergosterol daily for 2 days. |
| 238 | 0.41 | 1.64 | 3.28 | 2.82 | 1 " " " " 2 " |
| 239 | 0.44 | 1.32 | 3.31 | 2.42 | 1 " " " " 2 " |
| 241 | 0.45 | 1.87 | 3.06 | 1.76 | 1 " " " " 2 " |
| 242 | 0 86 | 1 45 | 3.02 | 3.46 | 1 " " " " 2 " |
| 245 | 0.88 | 1.53 | 3.46 | 3.16 | 1 " " " " 2 " |
| 247 | 0.56 | 1.11 | 2.98 | 3.12 | 1 " " " " 2 " |
| 249 | 0.69 | 1.50 | 2.96 | 3.20 | 1 " " " " 2 " |
| 268 | 0 97 | 1 32 | 3.14 | 3.03 | 5 min. ultra-violet light, two treatments 1st day, one 2nd day, sample taken after third treatment. |

TABLE III.

*Decrease of Serum Calcium in Cases of Spontaneous Healing.*

Values are expressed in millimols per 1000 cc. of serum.

| Experiment No. | P | Ca |
|---|---|---|
| 18 | 2.07 | 2.11 |
| 150 | 2 75 | 1.88 |
| 47 | 3.10 | 1.60 |

**P in normal rabbits, 2.86, standard deviation ±0.51; Ca in normal rabbits, 2.95, standard deviation ±0.31. These figures were obtained on a series of twenty-two rabbits of the same age as those used in the experiments and receiving a diet of oats and green leaves or the Steenbock stock diet (yellow corn, 76.0 gm.; gluten flour, 20.0 gm.; $CaCO_3$, 3.0 gm.; $CaCl_2$, 1.0 gm.).**

decrease of the serum calcium, while in three (also after 2 days) the calcium had increased. Of the animals treated with ultraviolet light only one is included in Table II, that being the only one in which the treatment resulted in an increase of the phosphorus. This animal shows a slight, but quite definite, decrease of the calcium after treatment.

That the treatment of rickets may result in tetany has been known since the publication of Huldschinsky (7) and recently confirmed by Gerstenberger and his collaborators (8). None of the rabbits in our series showed any clinical signs of tetany, but the drop in serum calcium (observed also by Gerstenberger) was occasionally very marked. We have observed the same phenomenon also in spontaneously healing rickets in rabbits (Table III). In a large series of rachitic rabbits, fourteen showed definite x-ray evidence of healing. Of these fourteen, three showed markedly diminished calcium concentration and normal values for phosphorus. One rabbit, showing no signs of healing, presented the same blood picture, but it must be remembered that not until healing has proceeded for some time can it be diagnosed by x-ray.

## DISCUSSION.

The decrease of the serum calcium accompanying a sudden increase of the serum phosphorus in rickets indicates that the serum, before the rise of the phosphorus occurred, held as much of calcium and phosphorus as was compatible with existing conditions. Before the phosphorus had, by one way or another, been made to increase, there was no relationship between the concentration of phosphorus and of calcium; the latter being always present in normal amounts, while the phosphorus was more or less markedly decreased, according to the severity of the rickets. After the phosphorus had increased, however, there was a definite relationship between the concentration of calcium and of phosphorus (Fig. 1); the higher the phosphorus, the lower the calcium. (The relationship is, obviously, a logarithmic one, and the correlation coefficient between log Ca and log P is −0.74 ± 0.13. The data are compatible with a compound as acid as $\mathrm{Ca}_{1\ 1}\mathrm{P}$ and as alkaline as $\mathrm{Ca}_{3\ 7}\mathrm{P}$.)

The results of our experiments show that the serum in rickets, in spite of the low concentration of phosphorus, still is saturated in the same way as the normal serum. It has already been pointed out that the conditions in the serum are not comparable to those obtaining in an inorganic solution, and when we use the word saturation in this connection we are well aware that this may not be saturation in the ordinary sense of the word. Our experiments and the similar experiments made on normal animals do not add anything to the question of how calcium and phosphorus are bound in the serum and we feel, therefore, that this is not the place to discuss the numerous *in vitro* experiments bearing on this question. It is possible that the decrease of the calcium may better be described as a threshold phenomenon, as the calcium must leave the blood stream either by way of the capillary wall or the kidneys, and this threshold phenomenon may or may not be connected with the solubility of calcium phosphate in the serum. If it is dependent on solubility, then the presence of this phenomenon in rickets, where the serum phosphorus is decreased, indicates that the factors governing solubility must in rickets be adjusted differently than in the normal serum. On the administration of cod liver oil, the increase of the phosphorus would, then, precede the readjustment of the solubility factors and the initial effect would be a depression of the calcium concentration. After a period of a few days the readjustment of the factors governing solubility would take place, allowing the serum to hold normal amounts both of

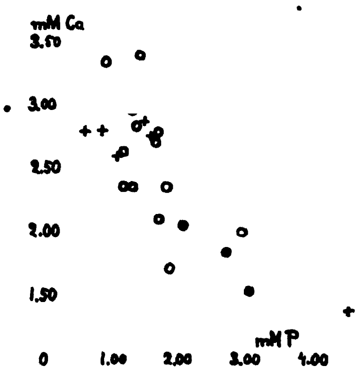

FIG 1. Relation between serum calcium and serum phosphorus after administration of phosphorus (crosses), antirachitic therapy (circles), and spontaneous healing (dots).

calcium and phosphorus. We are at present investigating the validity of this tentative hypothesis.

### *Methods.*

The rabbits were made rachitic with the McCollum Diet 3143 (9). They were put on this diet at the age of 4 weeks and the experiments made after they had been on the diet for 20 to 50 days.

The phosphorus was determined by the method of Fiske and Subbarow (10), calcium according to a procedure similar to that of Fiske and Logan (to be published).

## SUMMARY.

In rachitic animals an increase of the serum phosphorus (as a result of phosphorus administration, therapeutic agencies, or spontaneous healing) is often accompanied by a decrease of the serum calcium.

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# FURTHER STUDIES ON THE ISOMERIZATION OF ERGOSTEROL.

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There are many isoergosterols, and the literature pertaining to them is confused. For discussion, they may be classified into groups which are convenient, if not chemically systematic: (1) naturally occurring, activatable isomers. Examples are found in the ergosterols of different specific rotation, obtained by Heilbron, Sexton, and Spring (1) and by Bills and Cox (2) from yeast grown under different conditions. The existence of these isomers in difficultly separable mixtures is undoubtedly a factor in the lack of agreement as to the physical constants of ergosterol.

(2) Activatable isomers prepared from ergosterol. Examples are the ergosterols of different rotation and chemical properties obtained through acetylation by Heilbron and Sexton (3), and by Heilbron, Sexton, and Spring (1). Some of these forms may be identical with forms of the first group, to which they are at least closely related. Their existence has been questioned by Lettré (4). The esterification of ergosterol is always fraught with the possibility of isomerism, even though the preparation of ergosteryl isobutyrate by Bills and Honeywell (5) apparently involved no such change. By treatment of ergosterol with benzoyl chloride in pyridine, Windaus and Rygh (6) obtained a benzoate, $[\alpha]_D = -68°$, whereas Bills and Honeywell reported $[\alpha]_D = -177°$. Some slight difference in procedure had occasioned the great difference in rotatory power.

(3) Non-activatable isomers prepared from ergosterol. Examples are the isoergosterols obtained with inorganic acids, and with acid chlorides in the absence of the pyridine diluent (Reindel, Walter, and Rauch (7), Bills and Cox (2), Windaus and Rygh (6), Heilbron and Spring (8)).

In a previous paper Bills and Cox (2) described three isoergosterols of the third group which were prepared from ergosterol by the catalytic action of hydrochloric acid, hydrobromic acid, and cinnamoyl chloride. These forms, mutually transformable by the appropriate catalysts, were distinguished by their specific rotations. The question arises, are they chemically individual, or do they consist of mixed isomers in equilibrium?

It was shown that the three forms differed mainly in rotatory power; very little in melting point or crystal habit. We have since observed that the absorption curves are strikingly similar in shape and height, extending from about 220 to 270 $m\mu$, reaching a maximum at 248 $m\mu$, and indicating a molecular extinction coefficient of approximately 16,000.

In the present investigation the isomers were subjected to repeated crystallization from alcohol. Nearly saturated hot solutions were cooled gradually to room temperature, refrigerated a few hours, and the crystals filtered off. The crystals were dehydrated in a high vacuum at 80° for 10 minutes. The mother liquors were reduced to dryness by warming under a current of air, and the residues were then dehydrated like the crystals. All crystal crops and their mother liquors were examined polarimetrically, and most of them, spectrographically. The polarimetric determinations were made in chloroform; the spectrographic, in alcohol. Thus we were able to observe the trend of fractionation very closely.

A new supply of hydrochloric isomer was prepared. 20 gm. of purified ergosterol, $[\alpha]_{5461}^{25} = -157°$, were dissolved in 700 cc. of chloroform. 40 cc. of HCl, sp. gr. 1.18, were added, and the mixture was shaken in the dark for 6 hours at 25°. The chloroform layer was separated from the aqueous layer and rapidly evaporated over a boiling water bath. The residue was comminuted with 150 cc. of alcohol, refrigerated, filtered with suction, and washed with four 50 cc. portions of ice-cold alcohol. The product thus obtained was designated Crop 1. The combined filtrates were diluted with $\frac{1}{3}$ volume of water, and a fine precipitate was recovered from the yellow solution of decomposition products. This precipitate, still somewhat yellow, was designated Liquor 1. Crop 2 was obtained by recrystallizing Crop 1 from alcohol, the crystals being washed with a little ice-cold alcohol. The combined filtrates from Crop 2 were evaporated to dryness, giving Liquor 2. Successive crystallizations gave Crops 3 to 13 and Liquors 3 to 13.

The hydrobromic isomer was prepared like the hydrochloric, except that HBr, sp. gr. 1.39, was employed instead of HCl, and the time of shaking was reduced to 2 hours. Crops and Liquors 1 to 13 were obtained.

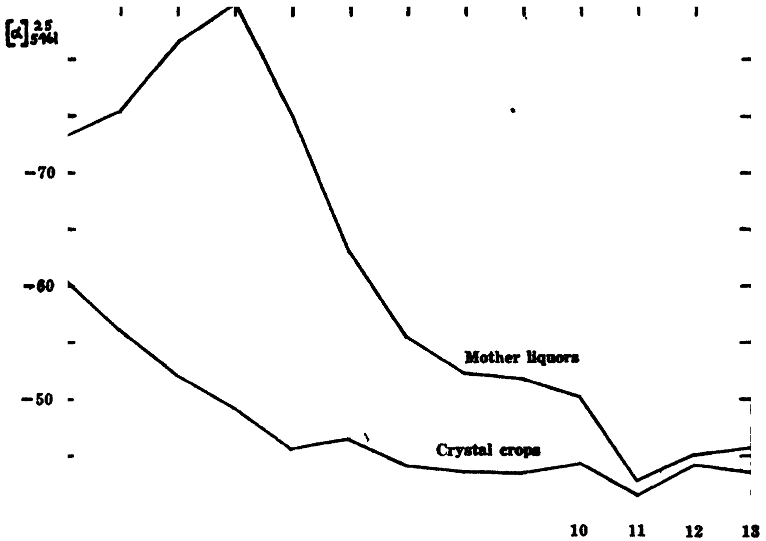

FIG. 1. Fractional crystallization of hydrochloric isomer. Weight of Crops 1 to 13, in gm.: 16.1, 14.0, 12.8, 11.2, 10.4, 9.6, 8.4, 7.8, 7.4, 6.6, 6.0, 5.2, 4.6.

Cinnamic isomer was prepared by heating equal parts of dehydrated ergosterol and cinnamoyl chloride[1] at 170° for 5 minutes. The product was dissolved in ether at room temperature and filtered through hardened paper. To the clear solution 1.5 vol-

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[1] Cinnamoyl chloride is unstable, even in the absence of moisture. Should commercial preparations be unsatisfactory, this reagent may be prepared by refluxing 10.0 gm. of cinnamic acid, 20.0 cc. of benzene, and 12.0 cc. of oxalyl chloride for 20 minutes, and then distilling off the benzene (reaction of Adams and Ulich).

umes of 96 per cent alcohol were added. The solution after standing overnight at $-16°$ was filtered; the cinnamate leaflets were washed with a little pure acetone, and twice recrystallized from permanganate-treated ligroin. 10 gm. of the cinnamate were refluxed with 500 cc. of 2 per cent KOH in 96 per cent alcohol. Boiling was continued for 5 minutes after the last crystal dissolved, and then to the hot solution 125 cc. of hot water were added. The product was refrigerated, filtered with suction, washed with 65 per cent alcohol, and then with 50 per cent alcohol until the filtrate was neutral to litmus. The fine, colorless, crystals comprised Crop 1. There was in this instance nothing to designate as Liquor 1. Crops and Liquors 2 to 11 were obtained by recrystallization.

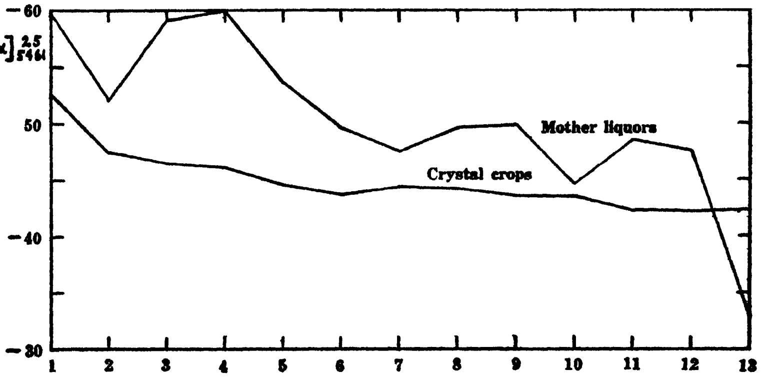

FIG. 2. Fractional crystallization of hydrobromic isomer. Weight of Crops 1 to 13, in gm.: 16.1, 14.4, 13.6, 12.4, 11.6, 10.8, 9.6, 9.2, 8.6, 7.6, 6.5, 6.0, 5.4.

The fractionation of the hydrochloric isomer is illustrated in Fig. 1. The first crude crystals, Crop 1, showed $[\alpha]_{5461}^{25} = -60.8°$. Successive crystal crops exhibited less and less levorotation, the decline being rapid and regular at first; less rapid and somewhat irregular towards Crop 13. Crop 13 gave $[\alpha]_{5461}^{25} = -43.6°$; m.p., 142°. The general form of the curve suggests that an infinite number of crystallizations would reduce the specific rotation to about $-41°$.

The first mother liquor gave $[\alpha]_{5461}^{25} = -73.1°$. As decomposition products were progressively removed there was a marked increase in optical activity, Liquor 4 showing the maximum $[\alpha]_{5461}^{25} = -85.0°$. From Liquor 4 to Liquor 13 the specific rotation decreased, approaching that of the crystal crops. It is possible that if no decomposition products had been present, Liquor 1 would have exhibited greater optical activity than Liquor 4.

The fractionation of the hydrobromic isomer is illustrated in Fig. 2. Crystal Crop 1 showed $[\alpha]_{5461}^{25} = -52.5°$. With successive crystallizations the rotation decreased with considerable regularity, the curve seeming to approach −41° as its limit. Crop 13 gave $[\alpha]_{5461}^{25} = -42.3°$; m.p., 142°. Liquor 1 gave $[\alpha]_{5461}^{25} = -59.8°$. Liquor 2 exhibited less optical activity, probably on account of a yellow decomposition product which was concentrated into it from Crop 1. Liquor 4 again showed the maximum levorotation, 59.9°. From Liquor 4 to Liquor 12 there was a definite, though somewhat irregular, decrease in rotation. Liquor 13 showed a sudden drop, of doubtful significance, since this product yellowed noticeably during evaporation. Changing the solvent to acetone resulted in no better separation of either the hydrobromic or hydrochloric fractions.

The fractionation of the cinnamic isomer is illustrated in Fig. 3. One should bear in mind that this fractionation actually began when the isoergosteryl cinnamate was crystallized from ether and ligroin. How much separation took place at those stages is unknown, since our curves illustrate only the fractionation obtained after the ester had been saponified.

Crystal Crop 1 showed $[\alpha]_{5461}^{25} = -40.2°$. With successive crystallizations the rotation decreased with considerable regularity, the curve seeming to approach −31° as its limit. Crop 11 gave $[\alpha]_{5461}^{25} = -32.7°$; m.p., 145°. The first liquor available, Liquor 2, gave $[\alpha]_{5461}^{25} = -72.9°$. Successive liquors exhibited, in general, less and less optical activity, the specific rotation approaching that of the crystal crops.

The original isoergosterol of Reindel, Walter, and Rauch (7) was prepared by passing hydrogen chloride through a chloroform solution of ergosteryl acetate, and saponifying the resultant isoergosteryl acetate. Heilbron and Spring (8) reported that the isoergosteryl acetate could be fractionally crystallized into two forms, which gave upon hydrolysis two isoergosterols, $[\alpha]_{5461}^{21} = -134.2°$

and $[\alpha]_{5461}^{21} = -95°$. Reindel's isoergosterol gave $[\alpha]_D = -87°$, which is equivalent to $[\alpha]_{5461} = -110°$. In our laboratory several attempts to prepare isoergosterol by the Reindel method yielded a product no more levorotatory than $[\alpha]_D^{25} = -40°$ or $[\alpha]_{5461}^{25} = -51°$. In one instance, by very brief treatment, we did obtain a mixture showing higher rotation, but this probably contained ergosterol, because it was activatable by ultra-violet light. We cite these data, not as a contradiction of the European workers, but as an example of the delicateness of molecular relations encountered in ergosterol chemistry.

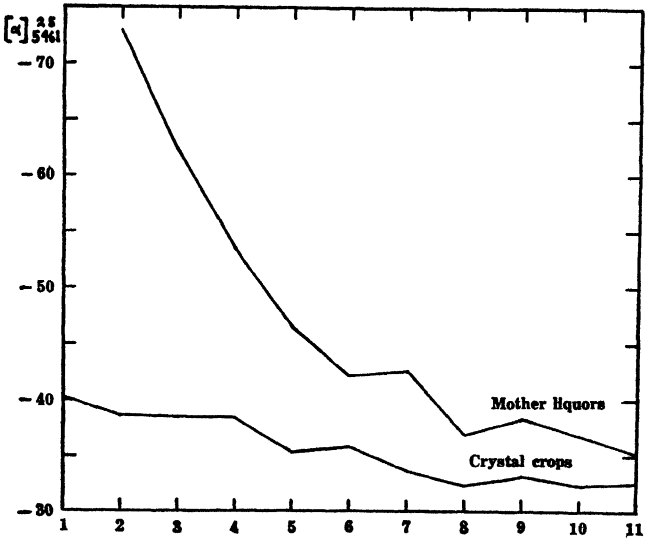

Fig. 3. Fractional crystallization of cinnamic isomer. Weight of Crops 1 to 11, in gm.: 7.1, 6.5, 5.7, 4.8, 4.4, 4.0, 3.7, 3.3, 3.1, 2.9, 2.4.

Heilbron and Spring further reported that by the action of alcoholic sulfuric acid on ergosterol a pure isoergosterol was obtained in almost quantitative yield. It appeared to be identical with one of the fractions, $[\alpha]_{5461}^{21} = -134.2°$, prepared from the Reindel acetate. It occurred to us that this might be the high rotating isomer which was present in the liquors from our hydrochloric, hydrobromic, and cinnamic isomers. To prepare a supply of it we followed the directions of Heilbron and Spring as closely as the published details permitted. 15 gm. of ergosterol, $[\alpha]_{5461}^{25} = -157°$, were refluxed for 1 hour with a mixture of 100 gm. of concentrated sulfuric acid made up to 1000 cc. with 96 per cent alcohol. 260 cc. of water were added, and the product was refrigerated. The crystals were filtered off and washed with 65 per cent alcohol until the filtrate was neutral to litmus. The nearly white product thus obtained was designated Crop 1. There was in this instance nothing to designate as Liquor 1. Crop 2 was obtained by recrystallizing Crop 1 from alcohol-benzene (2:1),the crystals being washed with 65 per cent alcohol. The combined filtrates from Crop 2 were evaporated, giving Liquor 2. Crops and Liquors 3 to 8 were obtained by successive crystallizations from alcohol.

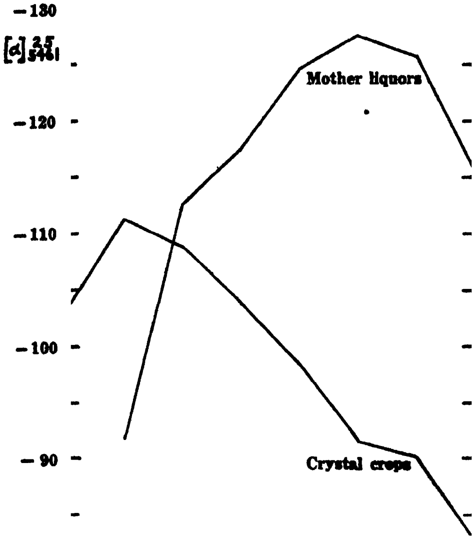

Fig. 4. Fractional crystallization of sulfuric isomer. Weight of Crops 1 to 8, in gm.: 11.1, 5.0, 4.1, 3.3, 2.9, 2.5, 2.0, 1.8.

The fractionation of the sulfuric isomer is illustrated in Fig. 4. To our surprize, this was not a pure compound, but just as much a mixture as the others. Crystal Crop 1, containing a trace of pigment, showed $[\alpha]_{5461}^{25} = -103.3°$. Crop 2, which was colorless, showed $[\alpha]_{5461}^{25} = -111.2°$. Successive crops decreased in specific rotation, Crop 8 showing $[\alpha]_{5461}^{25} = -82.6°$; m.p., 145°. The curve gives little indication of the ultimate rotation which an infinite number of crystallizations would bring; certainly that value would be much lower.

The first liquor available, Liquor 2, gave $[\alpha]_{5461}^{25} = -91.9°$. Successive liquors exhibited increasing levorotation, until Liquor 6 showed the maximum, $[\alpha]_{5461}^{25} = -127.6°$; m.p., 112°. Applying the proper temperature correction, we calculate $[\alpha]_{5461}^{21} = -130.8°$. This is practically the value given by Heilbron and Spring for their highly levorotatory "pure" isoergosterol. It is evident, from the liquor curve of Fig. 4, that our Liquor 6 was far from pure. If sufficient material had been available for a sub-fractionation of Liquor 6, we should have expected to separate it into a portion of comparatively low specific rotation, and a portion exhibiting a rotation many degress greater than the observed value. After Liquor 6 the specific rotations fell, Liquor 8 showing $[\alpha]_{5461}^{25} = -115.6°$. The fractionation was discontinued at this point, the remaining material being retained for conversion into hydrochloric isomer (see below).

It has already been shown, by Bills and Cox (2), that certain isoergosterols are intertransformable. Thus when one of the isomers (hydrochloric, hydrobromic, or cinnamic) is treated with the acid which produces another, the first is converted into the second. We have repeated this observation with our fractionated products. Crystal Crops 1,3 of the hydrochloric and hydrobromic isomers were united, and the mixture, $[\alpha]_{5461}^{25} = -43°$, was retreated with hydrochloric acid. Five successive crystallizations were made, the specific rotations of the crystals and liquors being determined as before. The fractionation curves were essentially identical with the curves for the original hydrochloric isomer.

Crystal Crop 8 of the sulfuric isomer was similarly treated with hydrochloric acid, and five crystallizations were made. In this case the fractionation curves were different from those of the original hydrochloric isomer. Each crystal crop showed about 6° less levorotation than the corresponding crop of the original. The liquors showed differences of from 8° to 21° less than the corresponding liquors of the original. Thus it appears that the mixture which we call the sulfuric isomer is not wholly intertransformable with the hydrochloric isomer, and presumably not with the hydrobromic or cinnamic isomers. It would seem that sulfuric acid exerts its isomerizing effect differently than the other catalysts. We are coming to realize that the isoergosterols produced by one or another catalyst are mixtures of isomers existing in equilibria determined by the catalyst. Possibly certain components of these mixtures are common, while others are specific to the catalyst.

Many absorption curves were plotted for the same fractions that were studied polarimetrically. The characteristic isoergosterol band was noted in all cases. The molecular extinction coefficient remained approximately 15,000 to 16,000 for the various crystal crops of the hydrochloric, hydrobromic, and cinnamic isomers. The extinction coefficient was lower—about 11,000—in Liquors 3 of the hydrochloric and hydrobromic isomers, where the specific rotation was high. Nevertheless, this lowering was not evident in Liquor 2 of the cinnamic isomer, where the specific rotation again was high.

The extinction coefficients of the sulfuric isomer fractions lent confirmation to the conclusion from polarimetric work, that this isomer is inherently different from the isomers produced by the halogen acids. The liquors showed high extinction coefficients, as well as high specific rotations. Liquor 5 gave $\epsilon = 18{,}000$; $[\alpha]_{5461}^{25} = -124.7°$. Conversely, Crystal Crop 8 gave $\epsilon = 9{,}000$; $[\alpha]_{5461}^{25} = -82.6°$. A small amount of apparently unchanged ergosterol was present in Crop 8, but not in the liquors.

The extinction coefficient of the product obtained by retreating Crops 13 of the mixed HCl-HBr isomers with HCl was characteristic of the hydrochloric isomer. The retreated sulfuric isomer, however, again gave evidence of its non-intertransformability. Its crystals and liquors both showed $\epsilon = 11{,}000$, a value which indicates that the sulfuric isomer was changed by the HCl, although not converted into the typical hydrochloric mixture.

## DISCUSSION AND SUMMARY.

It is evident that the isoergosterols produced by acid catalysts consist of several isomers in admixture. Partial separation of the component isomers can be effected by repeated crystallization, but complete separation would appear difficult of attainment. Our studies indicate that these isomeric mixtures may differ in kind, as well as in proportion, some of the components being mutually transformable with others, and some not.

The fraction showing the highest levorotation, $[\alpha]_{5461}^{25} = -127.6°$, was Liquor 6 of the sulfuric isomer; $\epsilon = 17{,}000$, m.p. = 112°. The fraction of lowest rotation, $[\alpha]_{5461}^{25} = -32.2°$, was Crop 8 of the cinnamic isomer; $\epsilon = 15{,}000$, m.p. = 144°. The fraction showing the highest molecular extinction coefficient, $\epsilon = 18{,}000$, was Liquor 5 of the sulfuric isomer; $[\alpha]_{5461}^{25} = -124.7°$, m.p. = 110°. The fraction of lowest coefficient, $\epsilon = 9000$, was Crop 8 of the sulfuric isomer; $[\alpha]_{5461}^{25} = -82.6°$, m.p. = 145°.

The lack of correlation between the specific rotation, molecular extinction coefficient, and melting point in the various preparations is evidence of the multiplicity of isomeric forms which must be present. While it is impossible to state exactly the number of forms, it seems to us that the data indicate the existence of at least five in the various isoergosterol mixtures.

The action of catalysts is so often similar to that of ultra-violet rays, that one might reasonably expect irradiated ergosterol to contain more than a single form of vitamin D. Actually, there is evidence in the recent investigations on chickens by Massengale and Nussmeier (9) of this laboratory, and clinical work in progress elsewhere, that activated ergosterol and cod liver oil are not identical in antiricketic action. Comparison of these two agents involves a consideration of isomerism.

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# THE DETERMINATION OF MANGANESE IN ANIMAL MATERIALS.*

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In a study of the manganese metabolism of rats it became necessary to analyze many animals for their manganese content. Serious interference, due to the precipitation of calcium sulfate, was encountered when the official periodate method was applied to these samples. The same difficulty had been experienced previously in the analysis of feedingstuffs (1), but in that work the calcium sulfate, formed during evaporatiom, was removed by filtration. This procedure did not work well when applied to the analysis of whole animals. There is so much calcium in the bones that a satisfactory concentration of the sample could not be accomplished without removing the calcium sulfate several times during the evaporation.

An attempt was made to remove all of the calcium sulfate at one time by adding ethyl alcohol to the solution until the mixture contained 70 per cent by volume of alcohol. While the calcium sulfate was completely precipitated by this procedure, distillation of the filtrate failed to get rid of all the alcohol, and, as a result, the development of the permanganate color proved very troublesome; three oxidations with periodate were sometimes necessary before the readings against the standard reached a maximum. There was also a tendency to form a brownish surface film, which made comparison with the standard difficult.

While the work was proceeding the paper of Davidson and Capen (2) appeared giving a modification of the official method in which hydrochloric acid is replaced by nitric, sulfuric, or phos-

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phoric acid, thus shortening the method and giving as good or better results. Upon trying phosphoric acid in the analysis of our animal materials, it was found that the interference of calcium salts was almost entirely overcome. The new method gave uniformly higher results and more complete recoveries of added manganese than the official method combined with the removal of calcium sulfate by alcohol.

The percentage of manganese in animal materials is so extremely small that more than 50 gm. of dry material must be taken in some instances to furnish 0.05 mg. of manganese. It is necessary to have at least this quantity of manganese in 25 cc. of solution if the color is to be read in a colorimeter, even of the long, prism type. Such a weight of material is unwieldy and requires many hours to ash. In our metabolism studies it was highly desirable to be able to obtain analyses on a 10 to 20 gm. sample. This quantity of material is sufficient if the solutions are compared in 50 cc. Nessler tubes. Eimer and Amend tubes No. 20466 with polished colorless glass discs which are fused on to the glass tubes are particularly satisfactory. If the column of solution is about 200 mm. in depth, 0.01 mg. of manganese in 50 cc. gives a readable color. The procedure finally adopted is as follows:

*Method.*—The porcelain dishes in which the material is to be ashed must be extracted with dilute HCl (1:4) prior to use. Ash the material in a muffle furnace at a cherry-red heat. To the contents of the dish add 5 cc. of sirupy phosphoric acid and sufficient distilled water (30 to 50 cc.) to permit thorough extraction of the ash, and heat on the water bath for about 30 minutes. Allow the dish to cool and then filter the extract through quantitative filter paper or manganese-free asbestos. Transfer the filtrate to a 250 cc. beaker, add approximately 0.3 gm. of $KIO_4$, and boil gently until the full development of the permanganate color takes place. Cool to about 40°, transfer to a Nessler tube, dilute to the mark, and mix by pouring back into the original beaker and again into the Nessler tube. Compare at once with a similarly treated standard of approximately the same strength. Two standards should be prepared and the one nearest the unknown should be used for comparison.

Fold a piece of white paper so that it forms an inverted V and reflects the maximum amount of light vertically and place on a larger sheet of white paper. Pour the lighter colored solution into one Nessler tube and while holding this and the empty tube over the reflector pour the stronger solution into the second tube until the colors of the two tubes match. With a rule graduated in mm. measure the column of liquid in each tube. Pour out some of the stronger solution and again match the two solutions. Readings should check within 2 or 3 mm. This simple method of matching the colors avoids the necessity of preparing a large number of standards.

TABLE I.

*Detailed Data Illustrating Procedure in the Determination of Mn in Tissues of the Rat.*

| Rat tissue. | Weight of sample dried at 100°. | Mn in standard. | Readings. | | Mn in sample. | Recovery of added Mn. | Mn |
|---|---|---|---|---|---|---|---|
| | | | Standard. | Sample. | | | |
| | *gm.* | *mg.* | *mm.* | *mm.* | *mg.* | *per cent* | *mg. per kg.* |
| Bone | 15 | 0.03 | 191 | 171 | 0.0335 | | 2.233 |
| " | 15 | 0.03 | 190 | 173 | 0.0330 | | 2.200 |
| " + 0.03 mg. of Mn | 15 | 0.03 | 187 | 182* | 0.0616 | 94.5 | |
| Hide | 20 | 0.02 | 155 | 197 | 0.0157 | | 0.785 |
| " | 20 | 0.02 | 167 | 187 | 0.0179 | | 0.895 |
| " + 0.03 mg. of Mn | 20 | 0.03 | 179 | 118 | 0.0455 | 95.7 | |
| Muscle | 15 | 0.01 | 173 | 197 | 0.0088 | | 0.587 |
| " | 15 | 0.01 | 176 | 200 | 0.0088 | | 0.587 |
| " + 0.03 mg. of Mn | 15 | 0.03 | 195 | 151 | 0.0387 | 99.7 | |
| Whole animal | 13.75 | 0.05 | 186 | 205 | 0.0453 | | 3.295 |
| " " + 0.01 mg. of Mn. | 13.75 | 0.05 | 229 | 209 | 0.0548 | 95.0 | |

* The sample was made to 100 cc. instead of 50 cc.

In case a cloudiness appears before the reading can be taken it may be removed by concentrating the solution to about 20 cc., transferring it to a Nessler tube, and allowing the precipitate to settle out as the solution cools. The clear solution may then be decanted off, reoxidized, and compared without any interference from turbidity.

The detailed data for three tissues of the rat and for one whole animal are given in Table I. No difficulties have been encountered in the analysis of other animal materials that have not been experienced in the analysis of these four. In fact, the analysis of bone represents the most difficult and tedious determination to which the method has been applied. Although it was necessary to remove the precipitate several times before oxidation could be accomplished without loss from spattering, a 94.5 per cent recovery of the added manganese was obtained. In this determination, as in all of our tissue analyses, the manganese for recovery was added prior to ashing. When the manganese content of the whole individual is desired this procedure cannot be employed because of the difficulty in obtaining representative samples of the dried body. In Table I the recovery on the whole animal was obtained by adding the manganese to an aliquot of the acid extract just prior to oxidation. A comparison of the percentages in the seventh column shows that the recovery of manganese is about the same regardless of whether it is added before or after the ashing.

TABLE II.

*Applicability of the Method to Some Typical Animal Materials.*

| Material. | Weight of sample. | Mn in sample. | Recovery of added Mn. |
|---|---|---|---|
| | *gm.* | *mg.* | *per cent* |
| Beefsteak, T-bone. | 20 | 0.0130 | 93.0 |
| Cheese, American | 34.2 | 0.0265 | 91.5 |
| Cod, fresh | 15 | 0.0095 | 104.5 |
| Eggs | 15 | 0.0165 | 103.5 |
| Lamb chops | 15 | 0.0185 | 91.5 |
| Lobster | 10 | 0.0252 | 91.8 |
| Milk | 412 | 0.0135 | 89.3 |
| Pork chops | 5 | 0.0062 | 95.8 |
| Shrimp | 15 | 0.0105 | 99.5 |
| Turkey, dark meat | 20 | 0.0315 | 88.5 |

That the method is applicable to a wide variety of animal materials is shown in Table II by the data obtained in the analysis of ten representative foodstuffs. Although the actual amount of manganese in these samples ranges from 0.0062 to 0.0315 mg., recoveries ranging from 88.5 to 104.5 per cent were obtained.

## SUMMARY.

The periodate method has been adapted to the determination of the small quantities of manganese found in animal tissue without the necessity of taking a large and unwieldy weight of sample. As little as 0.01 mg. of manganese has been satisfactorily determined. Good recoveries of added manganese were obtained.

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# THE DETERMINATION OF ARGININE IN DOG BLOOD.

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Arginine is the only known amino acid which possesses a guanidine group. It is the only guanidine derivative which is present in significant amounts in our diet, occurring in all proteins whose composition has been determined. For this reason, it has been considered as a possible precursor of creatine and the purines.

One phase of the metabolism of arginine is its hydrolytic decomposition by the enzyme arginase, present in the liver, into urea and the diamino acid, ornithine. The work of Thompson (1905) who studied the rate of urea excretion in dogs following the administration of arginine, and of Felix and Tomita (1923) on the rate of destruction of arginine perfused through the surviving liver of cats, indicates that the guanidine group of the greater part, at least, of ingested arginine is quickly split off. Whether a small portion of ingested arginine is metabolized in another manner, is not known. The specific character of the activity of arginase is illustrated by the paper of Felix, Müller, and Dirr (1928). Derivatives of arginine in which the carboxyl group is involved or in which the amino group is removed, no longer react with arginase. Therefore, if deaminization took place prior to the action of arginase on a portion of ingested arginine, the guanidine group would be protected against the action of arginase. In this connection may be mentioned the work of Bunney and Rose (1928), which indicates the ability of the animal organism to synthesize arginine as measured by the rate of growth in rats fed on diets from which arginine had been removed as completely as possible.

Abderhalden (1913) isolated arginine from blood. However, no methods have been available for studying the arginine content of the blood and tissues. The author (1930) reported a modification of Sakaguchi's reaction for arginine which is suitable for the colorimetric determination of arginine. Early in the work on the application of this reaction, it was noted that a Folin-Wu blood filtrate gave a color with this reaction. In this paper we have been mainly concerned with the substance present in dog blood which gives a color with the modified Sakaguchi reaction. Edlbacher, Krause, and Merz (1927) have reported that human blood contains the enzyme arginase while dog blood does not. For this reason, the values for arginase and arginine on human blood will be reported in a separate paper. The color given by dog blood filtrates could be due to incomplete precipitations of blood proteins, presence of polypeptides containing arginine, free arginine, or some guanidine derivative. None of the known constituents of blood filtrate gives a color with the Sakaguchi reaction.

The following experiments are offered to indicate that the substance responsible for this color reaction in blood filtrates is actually arginine and it may be determined in blood filtrates by means of the modified Sakaguchi reaction.

If 3 cc. of dog blood are diluted to 9 cc. with water and 0.02 cc. of argininase extract from liver is added, prepared according to the method of Hunter and Dauphinee (1930), and permitted to stand at room temperature for 30 minutes, the Folin-Wu filtrate of this blood gives a barely perceptible color. 95 per cent of the substance responsible for the color reaction was removed by this treatment. Blood samples permitted to stand the same length of time without the addition of arginase do not give any decreased color value. If arginine is added to dog blood and the blood then treated with arginase, the arginine added is likewise destroyed.

Table I shows the results of a series of dog blood samples treated in this manner. The arginase extract undiluted contained 100 units of arginase per cc., standardized by the method given by Hunter and Dauphinee. Dilutions of this arginase extract were made with water just before using. In every case the blood was drawn from the jugular vein from dogs that had received no food for at least 20 hours. 3 cc. of blood were diluted with 5 cc. of water and 1 cc. of the diluted arginase extract added. At the end of the specified time, 15 cc. of water were added and the blood precipitated with tungstic acid by the usual Folin-Wu procedure. One sample was precipitated immediately, another precipitated immediately after the addition of arginase, and another after standing 30 minutes without the addition of arginase. The values of this last blood are not included in Table I, since in no case was the color value of its filtrate less than that of the blood precipitated immediately. Values below 1.0 mg. per 100 cc. of blood could not be obtained directly but were estimated by adding 0.01 mg. (1 cc.) of arginine to 4 cc. of the blood filtrate and reading against a standard containing 0.015 mg. of arginine in 5 cc. These estimated values are probably correct to within 0.1 to 0.2 mg. per 100 cc.

TABLE I.

*Dog Blood Treated with Arginase (Liver Extract).*

| Experiment No. | Sex. | Weight. | Arginine content of blood. | | | | Arginase dilution. |
|---|---|---|---|---|---|---|---|
| | | | Control. | Length of time of arginase action. | | | |
| | | | | 0 | 10 min. | 30 min. | |
| | | *kg.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| 1 | F. | 19.5 | 3.85 | 3.80 | 0.62 | 0.13 | 1:20 |
| 2 | " | 14.2 | 2.66 | 2.56 | 0.45 | 0.13 | 1:20 |
| 3 | " | 14.4 | 2.60 | 2.65 | 0.55 | 0.14 | 1:50 |
| 4 | " | 20.3 | 2.61 | 2.60 | 0.65 | 0.14 | 1:50 |
| 5 | " | 9.6 | 3.33 | 3.40 | 1.96 | 0.37 | 1:100 |
| 6 | M. | 14.6 | 2.82 | 2.79 | 0.40 | 0.00 | 1:50 |
| 7 | " | 10.65 | 3.33 | 3.17 | 1.20 | 0.50 | 1:50 |
| 8 | " | 10.62 | 3.57 | 3.51 | 1.33 | 0.37 | 1:50 |
| 9 | " | 11.2 | 2.87 | 2.82 | 0.28 | 0.15 | 1:20 |
| 10 | " | 8.0 | 3.64 | 3.60 | 0.50 | 0.38 | 1:20 |

1 cc. of diluted arginase extract was used in every case.

A flavianate was also obtained from dog blood filtrate which had the crystalline appearance and physical properties of arginine monoflavianate. The following procedure was used in obtaining this flavianate.

5 liters of the Folin-Wu filtrate of dog blood, producing a color value with the modified Sakaguchi reaction equivalent to 14.7 mg. of arginine, were treated with 30 gm. of acid-washed Lloyd's

reagent (Hamilton, 1928). The Lloyd's reagent was reextracted with barium hydroxide and the barium removed from the filtrate with sulfuric acid. This filtrate gives a color value equivalent to 9.8 mg. of arginine. The filtrate was concentrated and treated with silver sulfate, the procedure given by Vickery and Leavenworth (1927) for the separation of histidine and arginine being employed. The substance responsible for the color appeared in the arginine fraction of the silver precipitate. The silver precipitate was dissolved in sulfuric acid and the silver removed with hydrochloric acid. The filtrate was concentrated to 20 cc. and 200 mg. of flavianic acid added. A precipitate was obtained which, on recrystallization twice from 10 cc. portions of water, presented the characteristic crystalline appearance of arginine monoflavianate. The weight of the flavianate was 18.9 mg., which corresponds to 6.7 mg. of arginine or a 68 per cent recovery of the substance from the Lloyd's extract as arginine monoflavianate. The precipitate did not melt when heated to 260°. Unfortunately, the physical properties of arginine monoflavianate do not permit a more definite identification. The color produced with the modified Sakaguchi reaction with equal amounts of the above flavianate and arginine monoflavianate was identical. The fact that the substance giving a color reaction in blood filtrate is destroyed by arginase and gives a flavianate similar to arginine, would indicate that this substance is arginine.

The values for the arginine content of dog blood (control sample) in Table I are within the range of the values encountered in the analysis of numerous samples of dog blood. The small residual color value of the filtrate from blood treated with arginase for 30 minutes, may be due to the incompleteness of the reaction, arginine to urea and ornithine, or to the presence of peptones or polypeptides containing arginine, or to some guanidine derivative. Apparently in normal dog blood at least 85 to 95 per cent of the color is due to arginine. Arginine, therefore, exists in significant concentrations in blood and may therefore play a part in the formation of substances in the animal organism for which the guanidine group of arginine may be required. Various hypotheses may be suggested as to the mechanism for the maintenance of this concentration of arginine in the blood, in spite of the known activity of the arginase present in the liver in destroying arginine.

Perhaps the most plausible explanation is that the hydrolysis of arginine by arginase at the concentration in blood is comparatively slow and is counterbalanced by the hydrolysis and liberation of arginine from proteins in the tissues. Glucose feeding causes a diminution of arginine in the blood and might be explained on the basis of its protein-sparing effect. Work is in progress on this point.

### *Method for Determination of Arginine in Blood.*

The solutions used in the determination of arginine in blood are the same as those described by the author (1930). An arginine stock standard containing 0.1 mg. of the base per cc. is made by dissolving 0.1209 gm. of arginine monohydrochloride in 1 liter of 0.1 N hydrochloric acid containing 0.2 per cent sodium benzoate. This solution keeps indefinitely. The dilute standard is made from this stock solution and is kept for not more than a day. For work on dog blood the most suitable standard is one containing 0.015 mg. of arginine per 5 cc.; *i.e.*, 3 cc. of the stock solution made up to 100 cc. with water.

The procedure is as follows: To 5 cc. of the Folin-Wu blood filtrate in a test-tube (150 mm. × 18 mm.) are added 1 cc. of 10 per cent sodium hydroxide followed by 1 cc. of 0.02 per cent $\alpha$-naphthol. The mixture is placed in an ice bath and permitted to reach a temperature of approximately 4–6°. Then 0.2 cc. of sodium hypobromite is added, the whole mixed thoroughly, and followed in 5 to 10 seconds by 1 cc. of 40 per cent urea solution; the solution is mixed and replaced in the ice bath. The standard is prepared in the same manner except that only 0.15 cc. of sodium hypobromite is used instead of 0.2 cc., as with blood filtrates. The tubes are not diluted to any specified volume, although this is permissible, and contain 8.2 and 8.15 cc. respectively. Comparisons should be completed in a colorimeter within 5 minutes after the addition of the sodium hypobromite. The extra 0.05 cc. of sodium hypobromite is used in the blood filtrate because of the presence of substances, such as urea, which destroy part of the hypobromite added. In a large series of determinations on blood filtrates from normal dogs, these proportions of hypobromite in standard and unknown have worked satisfactorily.

TABLE II.

*Comparison of Arginine Content of Dog Blood Precipitated at Various Dilutions.*

| Dog No | Sample No. | Dilution. | Arginine content. | | Variation from 1:100 dilution. |
|---|---|---|---|---|---|
| | | | *mg. per l. filtrate* | *mg. per 100 cc. blood* | *per cent* |
| 11 | 1 | 1:100 | 2.78 | 2.78 | |
| | 2 | 1:167 | 1.61 | 2.69 | −3.24 |
| | 3 | 1:250 | 0.91 | 2.26 | −18.70 |
| | 4 | 1:125 | 2.12 | 2.65 | −4.68 |
| 12 | 1 | 1:100 | 2.97 | 2.97 | |
| | 2 | 1:167 | 1.91 | 3.19 | +6.73 |
| | 3 | 1:71.5 | 4.14 | 2.96 | −0.34 |
| 13 | 1 | 1:100 | 1.94 | 1.94 | |
| | 2 | 1:71.5 | 2.61 | 1.87 | −3.6 |
| | 3 | 1:83.5 | 2.32 | 1.93 | −0.51 |
| | 4 | 1:167 | 1.18 | 1.97 | +2.04 |

TABLE III.

*Recoveries of Arginine Added to Blood.*

| Experiment No. | Type of blood. | Arginine added. | Arginine found. | Arginine recovered. | |
|---|---|---|---|---|---|
| | | *mg per 100 cc* | *mg per 100 cc.* | *mg. per 100 cc.* | *per cent* |
| 1 | Mixed rabbit blood. | 0.00 | 2.93 | | |
| | | 2.30 | 5.09 | 2.16 | 93.9 |
| | | 3.20 | 6.00 | 3.07 | 96.0 |
| | | 1.60 | 4.35 | 1.42 | 88.9 |
| | | 1.20 | 4.08 | 1.15 | 96.0 |
| 2 | Mixed dog blood. | 0.00 | 2.58 | | |
| | | 2.40 | 4.88 | 2.30 | 95.8 |
| | | 1.04 | 3.58 | 1.00 | 96.2 |
| | | 0.52 | 3.07 | 0.49 | 94.3 |
| | | 4.80 | 7.28 | 4.70 | 98.0 |
| | | 0.28 | 2.78 | 0.20 | 71.5 |
| | | 0.84 | 3.37 | 0.79 | 94.1 |

If the sodium hypobromite is over a week old, it is necessary to determine the amount required for complete color development before performing a quantitative test. In the case of blood with increased urea content, as in nephritis, the above proportions for hypobromite do not hold and if the urea content is too high a quantitative determination cannot be carried out.

To show the accuracy of the method for determining arginine in blood, the determination was carried out on blood precipitated at various dilutions and the values obtained compared with that observed on the filtrate of the blood obtained in the usual manner These results are shown in Table II. Dog 13 shows an unusually low value for normal dog blood. However, this dog was later found to be pregnant.

Sample 3 of Dog 11 gave an error of −18.7 per cent, due undoubtedly to the extreme dilution, giving a value of only 0.91 mg. of arginine per liter. The maximum error of the eight remaining determinations was +6.73 per cent, with an average error of 3.02 per cent, + or − signs being disregarded.

Table III gives the recoveries of arginine added to dog and rabbit bloods. The average recovery obtained was 92.5 per cent, which includes additions of arginine of 11 per cent of the original arginine value of the blood to 186 per cent.

## CONCLUSION.

The substance responsible in the Folin-Wu filtrate of dog blood for most of the color given with the modified Sakaguchi reaction is believed to be arginine, because it is destroyed by arginase (liver extract) and gives a crystalline precipitate with flavianic acid. A method is given by which arginine may be determined directly on the Folin-Wu filtrates of blood.

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# THE EXCRETION OF CHLORIDE IN ACHLORHYDRIA.

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In connection with studies of the morning alkaline tide in urine it was decided to carry out a series of determinations of urinary chlorides in patients in whom an absence of hydrochloric acid from the stomach had been demonstrated by the usual methods (1, 2). The results of forty tests upon twenty-four patients of this type form the basis of the present article.

The routine treatment of each subject was the same as that described previously (3). The patient was aroused at 7 o'clock and the night urine voided and discarded. Thereafter hourly specimens were collected through the morning. A meal consisting of a glass of milk, a glass of water, two slices of toast with butter, and an egg was given between 8 and 9 o'clock. The salt taken with this meal was not strictly controlled. The régime of the patients through the test period was not regulated in any way, but it is certain that they did not indulge in great activity. They were requested not to take water except at the morning meal, but it was fairly obvious that this request was not always observed. In most instances six specimens—the full number—were obtained. A few patients, however, did not collect the specimen for the last period. There were also a very few instances in which a single sample represented a 2 hour period. The chloride content was determined by the Volhard-Harvey method.

In Table I the average values and the interrelations of the values found in the different tests are given. Rates of excretion which tended to exceed the average occurred immediately after awakening and again between 11 and 12 o'clock. Water excretion tended to be low during the first part of the test, with the smallest values during the hour when the meal was eaten. The concentration of chloride in the urine clearly showed a maximum between 9 and 10 during the hour following the meal. The maximum corresponds to the chloride tide discussed by Bazzet and others (4), and to an excretion of the chloride retained during sleep studied by Simpson (5). The minimum occurring immediately after a meal has been mentioned specifically by Adolph (6) and considered by many other writers. It probably results from withdrawal of sodium chloride into the gastrointestinal tract. It is important to note that a decreased excretion of sodium chloride at this time takes place in patients who do not secrete hydrochloric acid into the stomach, and who do not, accordingly, show a decreased urinary acidity after the meal. A retention of sodium chloride at this time is therefore independent of two of the factors with which it is quite regularly associated in normal subjects. Simpson's (7) work which showed variations in chloride excretion on the 3rd day of a fast after the reaction of the urine had become constant also suggests that these factors are not necessarily related to each other.

TABLE I.

*Study of Average Values of Chloride Excreted.*

| Method of study. | Factor | Distribution by time. | | | | | |
|---|---|---|---|---|---|---|---|
| | | 7-8 a.m. | 8-9 a.m. | 9-10 a.m. | 10-11 a.m. | 11-12 a.m. | 12 a.m.-1 p.m. |
| Average rate. | Volume, *cc.* | 57 | 43 | 73 | 67 | 78 | 63 |
| | NaCl, *mg. per hr.* | 479 | 337 | 337 | 435 | 501 | 417 |
| | " " " *100 cc.* | 903 | 855 | 670 | 791 | 811 | 830 |
| Relative rate. | Volume, *units.* | 3.2 | 2.5 | 3.3 | 3.6 | 4.0 | 3.8 |
| | Cl rate, " | 3.6 | 2.6 | 2.3 | 3.8 | 4.4 | 3.7 |
| | " concentration, *units.* | 4.2 | 3.5 | 2.1 | 3.5 | 3.6 | 3.6 |

The method of calculating the units given in the last part of the table is explained in the text.

The second group of Table I is planned to show the relative rates of excretion in the different experiments. The method of calculation used in preparing it was as follows. The lowest value obtained in each test was called 1 and the highest 6. The numbers were tabulated according to the hours in which the lowest, highest, etc., values occurred. These were added and averaged. Such a method of expression diminishes the effect of unusually high and low values as markedly as average figures may exaggerate it, and should serve as a valuable check upon the calculations already given. It is evident that during the hour following the meal there was in most of the tests a rate of excretion and a urinary concentration of sodium chloride lower than those noted at any other time during the morning. The inference to be drawn from these results is therefore identical with that suggested by the average values.

The significance of the values obtained before the meal is not quite as conclusive. Undoubtedly there was usually a high concentration of chloride in the urine at that time, but the rate of excretion was not higher than it was when other samples were obtained. The high average rate of excretion between 7 and 8 o'clock is therefore influenced by the inclusion of experiments in which the rate was unusually high. The author believes, however, that the figures do show an increased excretion during this period. There was uniformly then a high chloride concentration, and the small volume of fluid which was passing through the kidneys has, he thinks, masked an actual increase in the excretion rate.

It seemed worth while to try to express the relative hourly metabolism of sodium chloride in some way which would exclude the effect of the rate at which fluid was excreted. Adolph (6) has shown that when the volume of urine is small the concentration of sodium chloride is nearly constant. The results in this series were strictly comparable with his when less than 50 cc. of urine were excreted in an hour. It was felt that if at any particular hour there was a concentration which was usually somewhat higher or lower than the average, a specific excretion or retention of sodium chloride was then taking place. When the urine is dilute the concentration varies inversely with the volume (8), and in our experiments when the water excretion exceeded 100 cc. per hour the rate of sodium chloride excretion was much more nearly independent of the water excretion, and showed a higher degree of constancy than did the chloride concentration. It was

## TABLE II.

*Distribution of Deviations of NaCl Values from the Mean.*

| Volume range. | NaCl factor studied. | Time of sample. | Deviation of NaCl values from mean. No. of cases. | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Under − 30 per cent. | − 21 to − 30 per cent. | − 16 to − 20 per cent. | − 11 to − 15 per cent. | − 6 to − 10 per cent. | − 5 to + 5 per cent. | + 6 to + 10 per cent. | + 11 to + 15 per cent. | + 16 to + 20 per cent. | + 21 to + 30 per cent. | Over + 30 per cent. |
| To 50 cc. | Concentration. | 7–8 a.m. | 1 | | | 1 | 2 | 8 | | 2 | 1 | 4 | 1 |
| | | 8–9 " | | 1 | | 1 | 6 | 9 | 3 | 3 | 1 | 1 | |
| | | 9–10 " | 2 | 3 | 1 | 3 | 3 | 4 | 2 | 1 | | | |
| | | 10–11 " | | 2 | 1 | 4 | | 9 | 5 | 1 | | | |
| | | 11–12 " | | 1 | | 1 | | 10 | 5 | 3 | 1 | | |
| | | 12 a.m.–1 p.m. | | | 1 | 1 | 2 | 6 | 6 | | 4 | | |
| Total | | | 3 | 7 | 3 | 11 | 13 | 46 | 21 | 10 | 7 | 5 | 1 |
| 51–100 cc. | Concentration. | 7–8 a.m. | | | | | | | 1 | 2 | 2 | | 1 |
| | | 8–9 " | 1 | 1 | | | 1 | 2 | 1 | 2 | 1 | | |
| | | 9–10 " | 1 | 2 | | 1 | 3 | 2 | 1 | | | | |
| | | 10–11 " | | | 1 | | | 3 | 2 | 1 | | | |
| | | 11–12 " | | | | 1 | | 3 | 1 | | | 1 | 1 |
| | | 12 a.m.–1 p.m. | | | 1 | | | 3 | | 1 | | | |
| Total | | | 2 | 3 | 2 | 2 | 4 | 13 | 6 | 6 | 3 | 1 | 2 |
| Over 100 cc. | Rate of excretion. | 7–8 a.m. | | | | | | | | | 1 | 2 | |
| | | 8–9 " | | | | | 1 | | | | | | 1 |
| | | 9–10 " | 3 | 1 | 1 | 2 | | | | | | | |
| | | 10–11 " | 1 | | | 1 | | 1 | 1 | | | | 1 |
| | | 11–12 " | | | | | 1 | 2 | | 1 | | | 2 |
| | | 12 a.m.–1 p.m. | 2 | | | | | 1 | | | | 1 | 1 |
| Total | | | 6 | 1 | 1 | 3 | 2 | 4 | 1 | 1 | 1 | 3 | 5 |
| Any. | Sum of above. | 7–8 a.m. | 1 | | | 1 | 2 | 8 | 1 | 4 | 4 | 6 | 2 |
| | | 8–9 " | 1 | 2 | | 1 | 8 | 11 | 4 | 5 | 2 | 1 | 1 |
| | | 9–10 " | 6 | 6 | 2 | 6 | 6 | 6 | 3 | 1 | | | |
| | | 10–11 " | 1 | 2 | 2 | 5 | | 13 | 8 | 2 | | | 1 |
| | | 11–12 " | | 1 | | 2 | 1 | 15 | 6 | 4 | 1 | 1 | 3 |
| | | 12 a.m.–1 p.m. | 2 | | 2 | 1 | 2 | 10 | 6 | 1 | 4 | 1 | 1 |
| Total | | | 11 | 11 | 6 | 16 | 19 | 63 | 28 | 17 | 11 | 9 | 8 |

When the value of a specimen was less than the mean the difference has been given a minus sign; when it was greater than the mean a plus sign has been used.

thought that if, under these conditions, maxima and minima of the rate of excretion occurred at any particular hour, they would indicate specific variations in sodium chloride metabolism at those times. In the intermediate range, when the volumes were between 50 and 100 cc., the concentration of the salt showed a somewhat greater constancy than did its rate of excretion, and it was therefore thought that a study of variations in the per cent of sodium chloride in specimens with volumes in this range might be of significance.

Table II gives the results of such an attempt to exclude the effect of water excretion upon chloride excretion. In preparing the first group the mean of the concentrations of all those specimens of each experiment with volumes less than 50 cc. was calculated. Specimens with volumes greater than this were disregarded. Next the per cent by which each specimen deviated from that mean was determined. The distribution of the deviations noted at different hours during the morning is given. It is evident that between 7 and 8 o'clock there tended to be many specimens with values which were unusually high, and that immediately after the meal there were many with unusually low concentrations. The process of calculation was repeated for the smaller number of specimens between 50 and 100 cc. with essentially the same results. In the third group there are reported deviations for specimens with volumes over 100 cc. Since in this range the excretion showed greater constancy than did the concentration the calculation was based on the former factor. Although the results were somewhat irregular, they show that the chloride content of the specimens collected before the meal tended to be higher, and that those obtained directly after the meal gave lower values than the average means. The summary given in the last part of Table II shows that if the effect of the water excretion is minimized as described, the excretion of chloride tends to be high immediately after waking and that the retention of this compound is rather marked immediately after the meal is fed.

## CONCLUSION.

In a series of cases of achlorhydria a low rate of excretion of sodium chloride during the hour following the morning meal can be clearly demonstrated. A relatively high rate of chloride excretion immediately after awakening can also be shown in such subjects. These variations seem to be independent of variations in the rate of water excretion.

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# A MICRO METHOD FOR THE ESTIMATION OF CHOLESTEROL BY OXIDATION OF THE DIGITONIDE.*

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The gravimetric procedure of Windaus (1) has for 20 years been considered the standard method for macro estimation of cholesterol. Because it is impossible to weigh accurately 1 to 2 mg. quantities of the cholesterol digitonide with ordinary analytical balances as well as because the digitonide is slightly soluble in and slowly precipitated from alcoholic solutions, this method has not been applicable to the rapid analysis of the small quantities of blood and tissue usually available for study.

The micro combustion with silver chromate and sulfuric acid recently developed by Bloor (2) for the estimation of other lipids has offered a method whereby the weighing of the digitonide can be avoided. Moreover, since the molecular weight of cholesterol, $C_{27}H_{46}O$, is 386, and that of cholesterol digitonide, $C_{82}H_{140}O_{29}$ is 1585, the oxidation to $CO_2$ and $H_2O$ of 1 mg. of cholesterol as such should require 3.92 cc. of 0.1 N $K_2Cr_2O_7$, while the oxidation of 1 mg. of cholesterol as digitonide should require 10.62 cc. of 0.1 N $K_2Cr_2O_7$.

Since the digitonide is a compound of constant composition, it has appeared to us that such a method may be expected to yield highly accurate results, even with very small quantities of cholesterol. Moreover, the chief contaminating glucoside in the commercially obtainable digitonin preparations, *i.e.* gitonin, has very

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† A large part of the work herein reported was done at The University of Rochester, while the writer was on leave from the University of California.

nearly the same solubilities and molecular weight as the digitonin itself; hence its presence should not appreciably affect the accuracy of the method.

Preliminary investigation has shown that the second objection, *i.e.* the slow precipitation of the digitonide, can be overcome by evaporation of the hot alcoholic cholesterol solution to dryness with the digitonin.

### *Separation of the Digitonide.*

The proposed determination must, however, be regarded as primarily a non-specific micro combustion of cholesterol digitonide. The chief problems involved are therefore concerned with separation of the precipitated digitonide from the other organic material present in the original blood and tissue extracts and from the excess digitonin used in the precipitation.

Cholesterol digitonide is, according to Windaus (1), soluble in cold 95 per cent ethyl alcohol to the extent of 4 mg. per 100 cc., soluble in boiling ethyl alcohol, methyl alcohol, glacial acetic acid, and pyridine. It is insoluble in pure ethyl ether, hot and cold water, acetone, and chloroform. Because of the relative solubilities of digitonin and of the lipids other than cholesterol present in blood and tissue extracts it has been found possible to avoid the lengthy and elaborate separation proposed by von Szent-Györgyi (3) and to free the digitonide from contaminating material by washing with alcohol-free ethyl ether and water only. But, in order to do this is has been necessary to design a small filter which is capable of being transferred bodily to the flask (a 125 cc. stoppered Pyrex Erlenmeyer flask) in which the final oxidation is to take place. A Pyrex glass tube 45 mm. long and 18 mm. in diameter, slightly constricted at one end, and fitted with a perforated porcelain plate cut from the bottom of a Gooch crucible and a pad of especially prepared asbestos, has met these requirements. This filter, fitted into a rubber stopper, may be handled as an ordinary Gooch crucible.

Attention should perhaps be called to the fact that digitonin is a saponin and its aqueous solutions have many of the characteristics of soap-suds. Hence separation of the digitonide from excess digitonin by centrifugalization from water solution is impossible. Moreover, the quantitative removal of the somewhat sticky plate-like crystals of digitonide from the sides of a flask and filter is impracticable, and it is very much easier to bring the oxidizing solution in contact with the filter and the inside of the flask in which the digitonide has been precipitated and washed. Although the mixture of Nicloux's reagent and N $K_2Cr_2O_7$ used in the final oxidation is not entirely stable at 124°, it has been found that the extra decomposition induced by the presence of the filter and asbestos is nearly uniform, comparatively slight (equivalent to 0.2 to 0.4 cc. of 0.1 N $K_2Cr_2O_7$), and easily corrected for by adding a washed asbestos pad and filter to the control flask.

Aside from the considerations mentioned above, the working out of the procedure for determination of free cholesterol given later in this paper has offered comparatively few difficulties. It has been possible to obtain very uniform recovery, both of pure cholesterol and of cholesterol added to plasma extracts. The reader is referred to Table I for figures.

### *Esterified Cholesterol.*

Since digitonin does not precipitate esterified cholesterol, it is necessary, in order to estimate the amount of cholesterol ester in a blood or tissue extract, to precede the precipitation of the digitonide by saponification. The greatest difficulties encountered in the adaptation of the digitonide method to cholesterol determination in plasma and in tissue extracts have been met with in finding the conditions under which saponification is complete, while the cholesterol is not decomposed. Cholesterol has been shown, in a long series of preliminary studies which space does not permit us to report, to be changed by strong alkali under the conditions imposed by the ordinarily used procedures for saponification in such a way that it is no longer quantitatively precipitable by digitonin. The method finally worked out is the result of a study of the conditions under which synthetic cholesteryl palmitate may be saponified with the least decomposition.

### *Cholesteryl Palmitate.*

The palmitate was prepared by heating a quantity (18 gm.) of pure cholesterol made by recrystallization from gallstones with the theoretical amount (12 gm.) of palmitic acid in an atmosphere of $CO_2$ at 200° for 4 hours. It was then separated from the uncombined palmitic acid and cholesterol by taking advantage of its comparative insolubility in boiling ethyl alcohol and recrystallized from alcohol ether mixtures until melting point determinations and analyses by saponification and direct combustion indicated that the preparation was to be regarded as a pure ester.

### *Saponification Procedures.*

The method for saponification described in detail under the heading of "Estimation of Total Cholesterol" has been adopted as the most practicable under ordinary laboratory conditions of those which have been found to give reasonably good recovery of the cholesterol. While space does not allow a report of the results of the long series of experiments which has led to this choice of method, certain things which have come to light during this study may not be omitted.

Cholesterol esters in general are to be regarded as difficult to saponify. Procedures which will secure complete hydrolysis require either fairly concentrated alkali or extremely long continued heating. Moreover, heating with alkali in the presence of air, even for the short time involved in the saponification procedure of Bloor *et al.* (4) for colorimetric estimation of cholesterol, has been shown to render from 10 to 40 per cent of the cholesterol in a 2 mg. sample of cholesteryl palmitate incapable of being precipitated by digitonin. Shortening the time of heating by removing the alcohol in a current of air has given slightly better but still unsatisfactory results. Passing a current of warm oxygen-free nitrogen through the flask during saponification of the ester has given about 95 per cent recovery of the cholesterol as digitonide. But this procedure involves so much extra apparatus and expense that it has been considered impracticable for ordinary use. Carbon dioxide has been substituted for the nitrogen with fairly satisfactory results (see Table I).

Extraction of the cholesterol from the residues left after saponification must be carried out *at once,* and, to secure the best results, *after* acidification. In general best results are secured if enough 1:3 sulfuric acid to neutralize the alkali used is added to the saponified residues the instant the last of the alcohol is removed.

| Nature of sample. | Amount of sample | No. of determinations | 0.1 N $K_2Cr_2O_7$ used. | Cholesterol found | Cholesterol recovered, per cent of theory. | Average variation from mean. | Type of procedure. |
|---|---|---|---|---|---|---|---|
| | | | *cc.* | *mg.* | | *per cent* | |
| Free cholesterol crystallized from petroleum ether. | 1.0 mg. | 13 | 10.26 | 0.963 | 96.3 | 0.8 | Oxidized on steam bath, 60 min. |
| | 0.5 " | 5 | 10.72 | 1.009 | 100.9 | 0.8 | Oxidized in oven 124° ± 2°, 15 min. |
| | 1.0 " | 10 | 10.53 | 0.993 | 99.3 | 3.0 | " " |
| | 1.5 " | 5 | 10.14 | 0.954 | 95.4 | 1.7 | " " |
| Cholesteryl palmitate. | 2 " | 15 | 9.94 | 1.15 | 93.1 | 1.7 | Saponified in current of warm N. Extraction from acid solution. |
| Plasma Extract I. | 10 cc. | 12 | | 0.93 | | 1.8 | Oxidized at 124°. |
| Same + 0.5 mg. cholesteryl palmitate.‡ | 10 " | 9 | 10.35* | 1.23† | 98* | 2.7† | " " 124°. |
| Cholesteryl palmitate. | 2 mg. | 9 | 9.98 | 1.16 | 93.6 | 1.8 | Saponification finished in $CO_2$. |
| Plasma Extract I. | 10 cc. | 4 | | 0.89 | | 2.1 | Extraction from acid solution. Oxidized at 124°. |
| Plasma Extract I + 0.5 mg. cholesteryl palmitate.‡ | 10 " | 4 | 9.93 | 1.17† | 91.6* | 0.7† | " " |
| Whole blood composite. | 10 " | 12 | | 0.95 | | 4.0 | " " |
| Cholesteryl palmitate. | 2 mg. | 9 | 9.13 | 1.06 | 85.7 | 5.9 | Saponified in current of air. Extraction from acid solution. Oxidized at 124°. |

*Chloroform* and *ethyl ether* have proved unsatisfactory for the extraction of cholesterol and fatty acid from this residue, probably because they are not absolutely immiscible with water. *Petroleum ether* on the other hand has proved very satisfactory in spite of the lower solubility of cholesterol in the solvent.

The nature of the change which renders the cholesterol incapable of precipitation with digitonide is not known. The fact that exclusion of air to a large extent prevents it would seem to indicate an oxidation. But there is also some evidence which points to an intramolecular rearrangement which leaves the alcoholic hydroxyl group in a position in which it is no longer free to combine with digitonin. However this may be, it seems worth while to call attention to the fact that the digitonin precipitation is to be regarded as a quantitative procedure for the precipitation of *unchanged sterol only* and that slight changes in the sterol molecule, such as are to be expected from heating with alkali at water bath temperatures, are by no means to be disregarded. Our experience with the purest cholesterol obtainable does not indicate that the mere addition of digitonin to a mixture of unsaponifiable material from an extract of plant or animal tissue means the removal of all the cholesterol derivatives originally present in the extract. Nor is it justifiable to conclude, with Lifschütz (5) that, because the unsaponifiable material from blood contains oxycholesterol and other cholesterol derivatives not precipitated by digitonin, these were necessarily present in the original blood. We have found that certain samples of supposedly pure commercial cholesterol prepared by saponification of brain tissue are not quantitatively precipitable by digitonin.

## *Specificity of the Digitonin Precipitation.*

Because of the importance which sterols other than cholesterol are assuming in biological studies as well as because of the considerations just stated, it seems worth while to list here the substances which react with digitonin. Windaus and his associates (6–8) have found that pseudocoprosterol and $\epsilon$-cholesterol, form no insoluble digitonides, while $\alpha$-naphthol, $\beta$-naphthol, $\beta$-bromonaphthol, carvomenthol $d,\alpha$-terpineol, $l,\alpha$-terpineol, and secondary octyl alcohol form with digitonin precipitates of varying degrees of insolubility. The only substances likely to be present in living tissue which are precipitable by digitonin are given by

Gardner and Williams (9) as cholesterol, stygmasterol, sitosterol, and possibly other phytosterols and coprosterol. Ergosterol has an insoluble digitonide, but oxycholesterol is only partially precipitated by digitonin.

### *Comparison with Colorimetric Procedures.*

We must probably always expect that a digitonin precipitation procedure will give lower values than a colorimetric procedure, not only for the reasons stated above, but because there is evidence that some of the products of decomposition of sterols give the color reaction with greater intensity than the original sterol itself (5). Which method gives a truer estimate of the sterol actually present is another question. In the case of blood and tissues which contain substances other than sterols producing color with acetic anhydride and $H_2SO_4$ the advantage of a method based upon a different principle is obvious. Twelve typical comparative analyses of a composite sample from normal whole blood have given, by the oxidative procedure 94 mg. of free cholesterol, and 190 mg. of total cholesterol per 100 cc. of blood; while by the colorimetric procedure of Bloor (4), the same extract has given 300 mg. of total cholesterol before and 240 mg. of total cholesterol after saponification. Ester cholesterol by the colorimetric procedure is 125 mg. Data from other comparative analyses are omitted because of lack of space.

The oxidative procedure is usually more time-consuming than the ordinary colorimetric methods for estimation of cholesterol. The writer has been able, in the course of a series of tissue analyses recently published, to make twenty-four cholesterol determinations in the average working day. A single determination requires about an hour.

While the separation of the digitonide requires a greater degree of analytical skill than the clinical methods now in use, the extra manipulation may, on the other hand be regarded as preferable to the uncertainty of the colorimetric estimation.

## *Procedure.*

### *Reagents Required.*

*1. Alcohol.*—Redistilled 95 per cent ethyl alcohol.

*2. Petroleum Ether.*—The fraction boiling at 40–60° is allowed to stand with frequent shaking over concentrated $H_2SO_4$ for 24 hours, and again redistilled.

*3. Ethyl Ether.*—The commercial anhydrous ethyl ether redistilled slowly.

*4. Normal Potassium Bichromate.*

*5. 0.1 N Sodium Thiosulfate.*

*6. 10 Per Cent Potassium Iodide.*

*7. 1 Per Cent Starch Solution.*

*8. Nicloux's Reagent.*—5 gm. of silver nitrate are dissolved in 25 cc. of distilled water in a 100 cc. centrifuge tube. To this are added 5 gm. of $K_2Cr_2O_7$ in 50 cc. of water. The precipitated silver bichromate is separated by centrifugation, washed twice with water by centrifugation to remove nitric acid, and the cake of precipitate dissolved without drying in 500 cc. of pure concentrated $H_2SO_4$.

*9. Sodium Hydroxide.*—Made by exposing metallic sodium to water vapor in a desiccator.

*10.* C. P. *Sulfuric Acid.*—(*a*) Concentrated and (*b*) 1:3 dilution.

*11. Carbon Dioxide.*—The ordinary commercial product from the tank filtered through cotton.

*12. Digitonin Solution.*—A 1 per cent solution of Merck's digitonin in 50 per cent alcohol.

*13. Asbestos.*—"Washed and ignited, medium fiber," commercial asbestos is heated overnight with $K_2Cr_2O_7$ in concentrated sulfuric acid, in the oven at 124°. The chromic-sulfuric acid is filtered off on a Buchner funnel, the asbestos washed with distilled water and sucked almost dry, covered with fresh chromic-sulfuric acid and heated as before. The procedure is repeated usually three times, and finally the asbestos is very thoroughly washed with distilled water, sucked dry, and suspended in distilled water.

### *Special Apparatus.*

*1. Filter Tube.*[1]—Pyrex glass tubing, 18 mm. in diameter, is cut in lengths of 45 mm. and slightly constricted at one end. Perforated porcelain plates cut from the bottoms of Gooch crucibles are fitted to these tubes—the whole functioning as a Gooch crucible, small enough to be placed in a stoppered 125 cc. Pyrex Erlenmeyer flask. This is used with the usual Gooch funnel and flask.

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[1] We are indebted to Mr. D. J. Kooyman of The University of Rochester for making these filter tubes.

2. An electric oven fitted with a $\frac{3}{8}$ inch iron plate, and capable or being regulated to maintain a temperature of 124° within 2°. The alternative is a steam bath which may be kept at 87°. A more detailed description of this apparatus is to be found in the paper of Bloor (2).

3. 125 cc. stoppered Pyrex Erlenmeyer flasks.

## *Determination.*

### *A. Preparation of Extracts.*

The alcohol-ether extracts of blood or plasma are made as described by Bloor (2) with a mixture of 1 part of ether to 3 parts of alcohol. The most convenient dilution has been found to be 5 cc. of blood or plasma to 100 cc. Where tissues other than blood have been used, we have weighed the minced tissue between watch-glasses, transferred it to a mortar, ground very thoroughly with sand, and transferred, as described by Bloor, with the addition of a small amount of water to a flask.

After boiling for 5 minutes with an amount of alcohol equal to about two-thirds of the final volume, the alcohol is poured off hot through a filter into a volumetric flask, and the residue boiled out repeatedly with small portions of ether which are poured off through the same filter. Finally the whole is made up to volume with alcohol and ether. This variation in the extraction procedure has been found advisable because of the very slight solubility of cholesterol esters in alcohol.

### *B. Estimation of Free Cholesterol.*

An aliquot of the extract containing about 0.5 to 1.2 mg. of cholesterol (usually 20 cc. in the case of plasma) is measured into a glass-stoppered 125 cc. Pyrex Erlenmeyer flask. 1 cc. of the 1 per cent digitonin solution is added and the whole evaporated just to dryness on the steam bath. The last traces of alcohol are removed with a current of air. The flask is taken from the steam bath and about 15 cc. of redistilled anhydrous ethyl ether are added *at once.*

The special filter tube with its pad of washed and oxidized asbestos is then prepared, washed with water and then with alcohol, and sucked almost dry. At this stage the minimum amount of suction should be used to avoid packing the asbestos too tightly.

The ether from the digitonide is poured off through this filter. The extraction of the digitonide is repeated three times more, the ether being heated to its boiling point each time. In each case the ether is poured off carefully though the filter. This ether extraction removes contaminating lipid material from the digitonide and must be thorough.

After the last of the ether is sucked through the filter, the filter tube and funnel are set aside and the filter flask emptied and washed out with cold water. This prevents back pressure of ether vapor during the subsequent washing with hot water.

The filter tubes and funnels are replaced; suction is increased gradually and the last traces of ether removed from the filter pads. The flask with the digitonide precipitate has meanwhile been covered with 15 to 20 cc. of distilled water and placed on the steam bath. This wash water, which contains in suspension most of the plate-like crystals of the digitonide, is poured off through the filter which has been used for the ether washings. A second portion of distilled water is added to the flask, and it is heated as before and poured off through the filter tube. It is usually necessary to make four washings with warm water. In all cases washing should be continued until the soapy appearance of the digitonin solution has entirely disappeared. The size and type of digitonide crystal obtained will differ considerably with different blood and tissue extracts. Usually the more lecithin present, the more difficult the removal, not only of lipid but also of excess digitonin.

After the last washing, when the filter is sucked dry, the tube and pad are removed from the funnel and placed bodily in the original stoppered Erlenmeyer flask, used for precipitating the digitonide.

The stirring rod which has been used in pouring the ether and wash water into the filter tubes, together with the mouth of the Erlenmeyer flask, is now washed carefully from a pipette with 1 cc. of C. P. concentrated sulfuric acid. The stirring rod is removed and 5 cc. of Nicloux's reagent and 3 cc. of N $K_2CR_2O_7$ are measured into the flask. At the same time a control flask is prepared containing filter tube, damp asbestos pad, 1 cc. of concentrated $H_2SO_4$, 5 cc. of Nicloux's reagent, and 3 cc. of N $K_2CR_2O_7$. At this stage the contents of the flask are agitated until the asbestos pads are separated into individual fibers. The flasks are loosely stoppered and heated in the oven at 124° for 15 minutes, or on the steam bath for 1½ hours. *Mixing the contents of the flasks so that the oxidizing agent comes in contact with every sq. mm. of surface is absolutely essential.*

After the heating, the contents of the flasks are immediately diluted with iced, distilled water to a volume of about 50 cc. 10 cc. of 10 per cent KI are added to each flask immediately before titration, and the free iodine is titrated with 0.1 N·$Na_2S_2O_3$ in the usual way, with a starch indicator.

*Calculation.*—The difference between the 0.1 N thiosulfate used for the control and that used for the unknown represents cc. of 0.1 N $K_2CR_2O_7$ used to oxidize the cholesteryl digitonide present.

1 mg. of cholesterol as digitonide requires 10.62 cc. of 0.1 N $K_2Cr_2O_7$ for complete oxidation. Hence:

$$\frac{\text{Cc. 0.1 N thiosulfate for control} - \text{cc. 0.1 N thiosulfate for unknown}}{10.62} =$$

mg. cholesterol in the aliquot used.

### *C. Estimation of Total Cholesterol.*

A second aliquot of the alcohol-ether extract (usually 10 cc. in the case of plasma) is measured into an ordinary 125 cc. Pyrex Erlenmeyer flask. If necessary, alcohol is added to bring the volume of actual alcohol present to 10 cc. Then 0.1 cc. of the strong NaOH solution is added, and the flask placed on the steam bath. When the volume of liquid in the flask has been reduced to about 1 cc., this is carefully distributed over the bottom of the flask and a stopper carrying a rather long inlet tube connected to a source of $CO_2$ and a shorter outlet tube is quickly put in place. The contents of the flask should be so adjusted that the stream of $CO_2$ will produce dryness in 1 minute. Heating in the $CO_2$ should not be prolonged. After drying, the flask is removed from the steam bath and an amount of 1:3 sulfuric acid, just greater than that required to neutralize the 0.1 cc. of NaOH, is added *at once*. While the flask is still hot, 15 to 20 cc. of purified petroleum ether are poured in, and the contents of the flask very thoroughly mixed by agitation. The petroleum ether is poured off through a small paper filter into the Erlenmeyer flask to be used for the oxidation, care being taken to prevent contamination with any of the aqueous layer in the flask. The extraction with petroleum ether is repeated four times in all—the flask being heated until the petroleum ether boils vigorously, and then thoroughly shaken, each time. This is necessary because of the relatively slight solubility of cholesterol in petroleum ether. Other solvents have not proved satisfactory, however, because even a trace of acid dissolved at this stage interferes with the subsequent precipitation of the digitonide.

The combined petroleum ether extracts are evaporated just to dryness, and the last traces of solvent are driven off with a current of air; the lipid remaining in the flask is dissolved in about 4 cc. of redistilled alcohol. 1 cc. of 1 per cent digitonin is then added, the contents of the flask evaporated to dryness, and the determination of the digitonide carried out exactly as for free cholesterol.

### *Total Unsaponifiable Matter (Optional).*

A third aliquot of the extract is measured out and saponified with 0.1 cc. of the NaOH exactly as for total cholesterol. The petroleum ether extraction of the residue is, in this case, carried out before neutralization of the $Na_2CO_3$. The petroleum ether extracts from this alkaline residue are, as before, poured off through a filter and evaporated to dryness in the oxidation flask. Instead of precipitating the cholesterol with digitonide, in this case, the entire residue is oxidized directly with the measured quantities of the oxidizing agent, and the factor used is 1 mg. of cholesterol = 3.92 cc. of 0.1 N $K_2Cr_2O_7$. Obviously this latter factor is only an approximation.

## SUMMARY AND CONCLUSION.

A micro procedure for the determination of free and total cholesterol by oxidation of the digitonide with silver chromate-sulfuric acid and subsequent titration of the excess dichromate with thiosulfate is described.

Cholesterol has been shown to be altered by the ordinary saponification procedures in such a way that it is no longer quantitatively precipitated by digitonin. A study of the conditions under which a synthetically prepared ester of cholesterol may be saponified with minimal alteration in the cholesterol has been made and a special procedure for saponification of blood and tissue extracts which conforms to these conditions has been evolved.

Attention is called to the fact that, because of this decomposition of cholesterol during saponification, many estimations of cholesterol by precipitation as digitonide from unsaponifiable matter have given results which did not represent the cholesterol originally present in the material.

The writer wishes to acknowledge her indebtedness to the Department of Biochemistry and Pharmacology at The University of Rochester, for the facilities which made this work possible, and to Dr. W. R. Bloor of that department, for his helpful advice and suggestions throughout its progress.

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# PLASMA LIPID LEVELS IN NORMAL DOGS IN THE POST-ABSORPTIVE STATE AND IN FASTING DOGS.

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In order to be able better to evaluate changes in plasma lipid levels occurring during diseased or experimental states it is necessary to have a clear conception of the variations that occur in the normal animal. The level of any hematological constituent depends on the relative rates of entrance and retirement from the circulating blood. Known physiologic factors which affect this ratio as applied to lipids are: (*a*) type of food ingested (1, 2), (*b*) fasting (3), (*c*) pregnancy (4), (*d*) menstruation (5) and reproductive cycle (6), (*e*) activity (7, 8), (*f*) age (9), (*g*) weight (10), (*h*) metabolic rate (2). It is obviously essential therefore carefully to evaluate interpretations of results in the light of the above. For these reasons it was considered desirable to undertake a controlled study of the normal variations in blood lipids.

In this study ten healthy male dogs, kept in heated rooms under standard conditions of exercise (5 to 10 minutes daily), maintained at a weight that varied less than 5 per cent, and fed a constant diet of biscuit,[1] were bled 15, 17, 19, 21, and 23 hours postabsorptive over the period of a month at intervals of 1 to 16 days. Samples of blood from the jugular vein were mixed with 3 drops of a saturated aqueous solution of sodium citrate, immediately centrifuged, and the plasma extracted. The analyses for total lipid and phospholipid were carried out on 5 cc. samples of plasma according to the methods outlined by Bloor (11, 12), his technique for the colorimetric determination of total cholesterol by the Liebermann-Burchard reaction also being used.

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[1] The composition of this biscuit was as follows: protein 17.91, fat 2.56, ash 8.60, crude fiber 2.14, and carbohydrate 61.81.

The accuracy and reproducibility of the methods in the hands of the investigator were determined by a series of analyses on known amounts of oleic acid and on alcohol-ether extracts of constant composition. The average of ten analyses of 2 mg. samples of oleic acid in petroleum ether gave a recovery of 99.4 per cent, ± 2.0 per cent from the gravimetrically prepared solution. Seven determinations of 0.5 mg. aliquots of cholesterol gave a recovery of 98.0 per cent ± 2.6 per cent. The cholesterol was obtained from human gallstones, recrystallized from alcohol and the last traces of spirit driven off by heating at 124° for 1 hour. The reproducibility of twelve total lipid analyses of a plasma alcohol-ether extract of constant composition containing approximately 2 mg. of lipid per sample, is shown by the low standard deviation of ± 1.4 per cent. Ten phospholipid determinations on an alcohol-ether extract of constant composition gave a standard deviation of ± 4.1 per cent with samples containing 2 mg. of lipid material. It was found that while the method for phospholipid was reproducible on stock alcohol-ether extracts of blood and tissues in the laboratory and on purified solutions of phospholipid in petroleum ether, when applied to plasma extracts from the dogs under study, comparatively low values which could not be duplicated were obtained. These unexpected and peculiar findings were checked and are being investigated by Dr. Bloor.

The reliability of the total lipid and cholesterol procedures as outlined by Bloor was verified. If the expression $\sigma = \sqrt{\frac{\Sigma x^2}{n}}$ is used to express the standard deviation, and the relation of that expression to the frequency curve as outlined by Dunn (13) is accepted as applicable to these results, then a variation of ± 1.4 per cent would encompass 68 per cent of the determinations of total lipid and limits of ± 2.6 per cent would include the same percentage of cholesterol values. Inasmuch as the total fatty acid value is computed by subtracting from the total lipid the amount of total cholesterol found, the accuracy for the total fatty acid figure cannot be greater than that of the cholesterol. Thus a difference between averages of more than ± 5.1 per cent (twice the standard deviation) would be a variation greater than that found in 95 per cent of the individual values obtained for either series, and could be safely termed significant. Significant changes in lipid levels were taken as average differences of more than ± 5 per cent.

Each postabsorptive 15 hour level is the average of four such determinations taken at intervals of 1 to 16 days. The total cholesterol levels (Table I) ranged from 68 mg. per cent in the youngest dog to 188 mg. per cent in the oldest. While each postabsorptive level varied only ± 6 per cent from the average of that particular dog, each mean varied by a standard deviation of ± 28 per cent from the average of the means. The total fatty acid levels (Table I) for each animal varied by a standard deviation of ±7 per cent from the mean of four determinations. These means however, ranged from 205 mg. per cent to 348 mg. per cent, with a standard deviation of ± 13 per cent.

TABLE I.

*15 Hours Postabsorptive Variations in Individual Total Fatty Acid, Total Cholesterol, and Total Fatty Acid: Total Cholesterol Values and Their Means.*

| Dog No. | Weight. | Height | Type. | Total fatty acid. | | * Total cholesterol. | | Total fatty acid: total cholesterol. | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean. | σ | Mean. | σ | Mean. | σ |
| | *kg.* | *cm.* | | *mg. per cent* | *per cent* | *mg. per cent* | *per cent* | | *per cent* |
| 29-288 | 19.0 | 103 | Police. | 212 | 8 | 68 | 6 | 3.12 | 4 |
| 29-176 | 32.3 | 117 | " | 205 | 8 | 81 | 14 | 2.64 | 18 |
| 29-225 | 20.0 | 100 | Collie. | 243 | 5 | 93 | 4 | 2.62 | 7 |
| 29-209 | 24.8 | 105 | Pointer. | 243 | 10 | 98 | 6 | 2.47 | 7 |
| 29-199 | 24.4 | 109 | Coach. | 270 | 8 | 108 | 6 | 2.49 | 3 |
| 29-203 | 25.6 | 111 | " | 297 | 8 | 115 | 11 | 2.58 | 3 |
| 29-210 | 20.7 | 102 | Pointer. | 240 | 2 | 124 | 2 | 1.92 | 1 |
| 29-207 | 20.8 | 103 | Coach. | 302 | 6 | 138 | 4 | 2.18 | 6 |
| 29-196 | 19.7 | 103 | " | 348 | 7 | 139 | 4 | 2.48 | 5 |
| 29-228 | 19.0 | 111 | Collie. | 348 | 5 | 188 | 2 | 1.85 | 3 |
| Average.......................... | | | | 271 | 7 | 115 | 6 | 2.43 | 6 |
| Standard deviation, *per cent*.... | | | | ±13 | | ±28 | | ±14 | |

Each mean represents the average of four determinations.

In addition to observing the 15 hour level, the total fatty acid and total cholesterol in the plasma on the 17th, 19th, 21st, and 23rd postabsorptive hours were determined on five dogs, with three series of observations on four animals and two series on one; a total of fourteen series. The slight variation from the average total fatty acid level is indicated by the average standard deviation of ± 4.1 per cent for the five dogs (Table II). The total cholesterol values (Table III) showed even greater constancy, having an average standard deviation of ± 3.7 per cent.

TABLE II.

*Variations of Total Fatty Acid Levels in Individual Dogs.*

| Dog No. | Postabsorptive hrs. | | | | | Mean. | Standard deviation. |
|---|---|---|---|---|---|---|---|
| | 15 | 17 | 19 | 21 | 23 | | |
| | | | | | | *mg. per cent* | *per cent* |
| | 194 | 179 | 191 | 182 | 194 | 188 | ±3.3 |
| 29-176 | 189 | 191 | 192 | 207 | 189 | 195 | ±3.5 |
| | 206 | 205 | 196 | 278 | 207 | 218 | ±15.0 |
| | 256 | 258 | 249 | 256 | 266 | 257 | ±2.1 |
| 29-199 | 256 | 258 | 260 | 254 | 269 | 259 | ±2.0 |
| | 269 | 261 | 257 | 266 | 265 | 263 | ±1.6 |
| | 230 | | 221 | 251 | 235 | 244 | ±6.0 |
| 29-203 | 284 | 277 | 270 | 298 | 278 | 281 | ±3.3 |
| | 276 | 278 | 285 | 286 | 286 | 282 | ±1.5 |
| 29-207 | 274 | 287 | 292 | 316 | 299 | 293 | ±4.7 |
| | 311 | 299 | 282 | 318 | 312 | 304 | ±4.2 |
| | 351 | 331 | | 350 | 325 | 339 | ±3.4 |
| 29-196 | 354 | 342 | 329 | 351 | 357 | 346 | ±2.9 |
| | 304 | 305 | 323 | 342 | 330 | 321 | ±4.6 |
| Average | | | | | | | ±4.1 |

It is evident, therefore, that the total cholesterol and total fatty acid levels in an individual dog at 15, 17, 19, 21, and 23 hours postabsorptive are practically constant over a period of 1 month of controlled conditions, the standard deviations for the series being less than the smallest significant difference. It is also apparent that between different dogs, there may be marked differences in the levels of those substances. It is interesting to note that the lowest values for both total cholesterol and total fatty acid were given by the youngest animals, while the highest values were obtained from the oldest animal. There was unfortunately no quantitative control of age. These findings are in accord with blood serum variations of total cholesterol with age, reported by Parhon and Parhon (9) in human beings. If the values are arranged in their order of magnitude, the sequence for total fatty acid and total cholesterol are practically the same. This parallelism between total fatty acid and cholesterol has been mentioned by Mayer and Schaeffer (2) in reference to the lipid levels in different species.

TABLE III.

*Variations of Total Cholesterol Levels in Individual Dogs.*

| Dog No. | Postabsorptive hrs. | | | | | Mean. | Standard deviation. |
|---|---|---|---|---|---|---|---|
| | 15 | 17 | 19 | 21 | 23 | | |
| | | | | | | *mg. per cent* | *per cent* |
| 29-176 | 60 | 59 | 56 | 57 | 60 | 58 | ±2.5 |
| | 95 | 91 | 84 | 84 | 86 | 92 | ±6.4 |
| | 74 | 73 | 72 | 92 | 72 | 73 | ±11.7 |
| 29-199 | 105 | 102 | 106 | 103 | 102 | 104 | ±1.6 |
| | 99 | 95 | 92 | 94 | 101 | 96 | ±3.5 |
| | 113 | 107 | 109 | 106 | 105 | 108 | ±2.8 |
| 29-203 | 86 | 85 | 84 | 83 | 81 | 85 | ±2.0 |
| | 108 | 105 | 102 | 102 | 102 | 104 | ±2.3 |
| | 105 | 106 | 105 | 102 | 100 | 104 | ±2.2 |
| 29-207 | 138 | 110 | 132 | 132 | 131 | 129 | ±7.4 |
| | 131 | 130 | 134 | 133 | 131 | 132 | ±1.1 |
| 29-196 | 135 | 134 | | 123 | 123 | 129 | ±4.5 |
| | 137 | 140 | 132 | 134 | 133 | 135 | ±2.2 |
| | 137 | 131 | 131 | 131 | 131 | 132 | ±2.4 |
| Average | | | | | | | ±3.7 |

Attempts to correlate metabolism (as indicated by surface area) and plasma lipid levels yielded ratios that were 2 and 3 times more variable than the individual levels themselves. The surface area formula $SA = 2.268 W \times L$ published by Cowgill and Drabkin (14) was used.

Terroine (3), working with dogs of unspecified sex, fed a diet of bread and lean meat, and bled from the left ventricle 36 hours postabsorptive over periods from 2 days to 11 months, analyzed whole blood, using the macro method of Shimidzu for total lipid, and the Windaus digitonide precipitation for cholesterol. His results, expressed in terms of per cent of dry weight, while not strictly comparable, are nevertheless interesting from the point of view of relative variations in the total fatty acid, total cholesterol, and total fatty acid : cholesterol ratios. If the standard deviations are computed from his published figures (Table IV) it is noted that the average of the standard deviations on individual dogs was ± 10.4 per cent for total fatty acid, ± 10.1 per cent for total cholesterol, and ± 4.0 per cent for cholesterol:total fatty acid ratio. These values show a slightly greater variation than those of the writer on plasma extracts. While it is obvious in Terroine's figures that there is less variation in his cholesterol:total fatty acid ratio, those ratios for plasma extract offer very little advantage over the average standard deviations of either total fatty acid or total cholesterol, which are ± 7.0 per cent and ± 6.0 per cent as compared to that for the total fatty acid:total cholesterol ratio of ± 5.8 per cent (Table I).

TABLE IV.

| Dog No. | No. of values. | Total fatty acid. | | Cholesterol. | | Cholesterol: total fatty acid. | |
|---|---|---|---|---|---|---|---|
| | | Mean. | Standard deviation. | Mean. | Standard deviation. | Mean. | Standard deviation. |
| | | | *per cent* | | *per cent* | | *per cent* |
| 1 | 7 | 1.527 | 10.5 | 0.485 | 12.7 | 0.310 | 4.7 |
| 2 | 3 | 1.370 | 12.9 | 0.439 | 11.2 | 0.316 | 3.0 |
| 3 | 6 | 1.431 | 8.4 | 0.383 | 9.8 | 0.260 | 5.5 |
| 4 | 2 | 2.001 | 9 9 | 0.478 | 6.8 | 0.245 | 3.0 |
| Average.................. | | | ±10.4 | | ±10.1 | | ±4.0 |
| Average of 14 dogs.. .. ...... | | 1.653 | ±27.4 | 0.590 | ±21.8 | 0.360 | ±20.0 |

Calculated from Terroine's published figures.

In general, the constancy of the plasma total fatty acids and total cholesterol in dogs is a reflection of the constancy of those elements in the blood of the rabbit as found by Iscovesco (15) and Horiuchi (16).

## SUMMARY.

1. In each of ten dogs the 15 hour postabsorptive plasma levels varied for total fatty acid by the standard deviation of ± 7.0 per cent. The total cholesterol varied by the standard deviation of ± 6.0 per cent, and the ratio between total fatty acid and total cholesterol by a standard deviation of ± 5.8 per cent.

2. The total fatty acid plasma levels in each of ten dogs over a period from the 15th to the 23rd hour postabsorptive varied by the sigma of ± 4.1 per cent and the total cholesterol by a sigma of ± 3.7 per cent.

3. The total fatty acid plasma levels in ten dogs under controlled conditions varied from 205 mg. per cent to 348 mg. per cent with a mean of 271 mg. per cent ± 13.0 per cent. The total cholesterol plasma levels varied from 68 mg. per cent to 188 mg. per cent with a mean value of 115 mg. per cent ± 28 per cent.

4. The highest values for total fatty acid and total cholesterol were obtained from an old dog, and the lowest values were given by young dogs.

It is a pleasure for the author to express his gratitude to Doctor Bloor for the advice and helpful criticism given throughout this investigation.

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# THE STRUCTURE OF GLUTATHIONE.*

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(Received for publication, May 16, 1930.)

The recognition by Hopkins (1) and by Kendall, McKenzie, and Mason (2), independently and almost simultaneously, that glutathione is a tripeptide, and contains glycine in addition to the previously recognized glutamic acid and cysteine, has once more thrown open the question of its structure. Disregarding questions of optical activity, twelve tripeptides of these three amino acids are possible. Six of these may be excluded at once on the basis of the evidence of Quastel, Stewart, and Tunnicliffe (3) confirmed by Kendall, McKenzie, and Mason (2), that the free amino group is that of glutamic acid.

The observation of Hopkins (1) that glutathione, when boiled with water for several hours, yields the diketopiperazine glycylcysteyl anhydride, is important, and, it seems to the writer, crucial, in demonstrating that glycine and cysteine are linked together in the original peptide. For this reason, the two structures[1] which involve the linkage of glycine and cysteine each to a separate carboxyl of glutamic acid, must be eliminated.

A study of oxidation products of glutathione has led to the general agreement (2, 3) that the γ-carboxyl of glutamic acid is involved in peptide formation. In connection with the discussion in the preceding paragraph, this would imply that the α-carboxyl is not so involved. There appear, then, to remain only two

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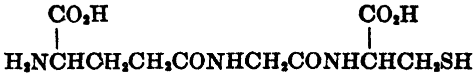
* The material contained in this paper was presented before the Division of Biological Chemistry at the Atlanta meeting of the American Chemical Society, April, 1930.

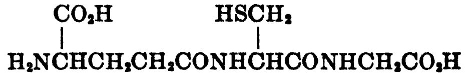
[1] One of these, $HO_2CCH_2NHCOCH(NH_2)CH_2CH_2CONHCH(CO_2H)$-$CH_2SH$, has been tentatively selected by Kendall and his associates (2) as representing glutathione; but this choice was made at a time when the results of Hopkins were presumably not available to them.

probable structures for glutathione. It must be either γ-glutamylglycylcysteine (I) or γ-glutamylcysteylglycine (II). A definite decision in favor of Formula II is made on the basis of the following new observations.



$$\overset{\displaystyle CO_2H}{\overset{|}{H_2NCH}}CH_2CH_2CONHCH_2CONH\overset{\displaystyle CO_2H}{\overset{|}{CH}}CH_2SH$$

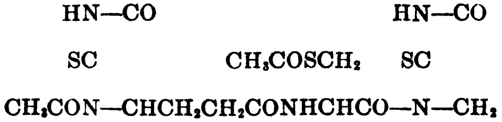
I.



$$\overset{\displaystyle CO_2H}{\overset{|}{H_2NCH}}CH_2CH_2CONH\overset{\displaystyle HSCH_2}{\overset{|}{CH}}CONHCH_2CO_2H$$

II.

Following a procedure suggested by the work of Schlack and Kumpf (4),[2] glutathione was condensed directly with ammonium thiocyanate and acetic anhydride according to the usual method (5) for the preparation of thiohydantoins from amino acids. The product, obtained in 77 per cent yield, was a yellow solid which it has not yet been possible to recrystallize, but which gives analytical data consistent with the expected formula (III) for a bis-thiohydantoin. The acetyl group attached to sulfur only



$$\begin{array}{llll} & HN\text{—}CO & & HN\text{—}CO \\ & SC & CH_3COSCH_2 & SC \\ CH_3CON\text{—} & CHCH_2CH_2CONH & CHCO & \text{—}N\text{—}CH_2 \end{array}$$

III.

increases the analogy between Formula III and the thiohydantoin (IV) of cysteine, which will be described in a subsequent paper.

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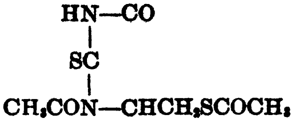
[2] Schlack and Kumpf have developed, on the basis of the investigations of Johnson (5) and his associates, a very promising method, hitherto lacking, for the identification of amino acids having terminal carboxyl groups in peptides. They have, however, made certain reservations as to the application of the process.

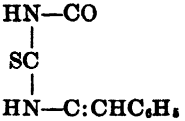
For instance, if the peptide is not previously benzoylated, they report that it is not in general feasible to isolate the peptide thiohydantoin. Further, as thiohydantoins derived from cystine have not been described, no peptides derived from this amino acid have hitherto been studied by their method.



$$\begin{array}{l}\text{HN—CO}\\ \;\;|\\ \;\text{SC}\\ \;\;|\\ \text{CH}_3\text{CON—CHCH}_2\text{SCOCH}_3\end{array}$$

IV.

The bis-thiohydantoin was condensed with benzaldehyde (6) and the product, after treatment with alkali and reacidification gave benzalthiohydantoin (V) in more than 50 per cent yield.



$$\begin{array}{l}\text{HN—CO}\\ \;\;|\\ \;\text{SC}\\ \;\;|\\ \text{HN—C:CHC}_6\text{H}_5\end{array}$$

V.

This product was thoroughly identified. Its formation is conclusive evidence that the glycine grouping in glutathione has taken part in the formation of one of the rings in Formula III. This, in turn, requires that in the original glutathione, the carboxyl group of glycine must have been free. The decision is thus clearly in favor of Formula II,[3,4] and against Formula I.

It is fully recognized that all the new results thus far reported in this paper would be equally consistent with a formulation of glutathione which attached the glycine and cysteine residues each

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[3] Attention should be called to the fact that Pirie and Pinhey (7), on the basis of dissociation constants obtained in the electrometric titration of glutathione, chose for the latter Formulas I or II, and recorded a slight preference in favor of Formula II.

[4] In view of the fact that the bis-thiohydantoin derivative (III) has an indefinite melting point, and does not lend itself to purification by recrystallization, it was preferred not to base the choice of structure for glutathione on this compound, except to the extent of the demonstration that 2-thiohydantoin is one of its hydrolysis products.

It is, however, of interest to point out that the analyses of this bis-thiohydantoin agree with the values calculated for the product most reasonably to be expected from a glutathione of the formula chosen. Moreover, the three conditions that (*a*) reduced glutathione should yield a bis-thiohydantoin; (*b*) the latter should, as demonstrated, yield 2-thio-4-benzalhydantoin; and (*c*) glycine and cysteine should be united in peptide linkage; lead to the choice of the structure selected above for glutathione without any additional assumptions.

to a separate carboxyl of glutamic acid. As has already been stated, such possibilities have been discarded on the sufficient basis of Hopkins' observation of the formation of a diketopiperazine derived from glycine and cysteine. It may be added, however, that the action of alkali on the thiohydantoin (IV) takes a rather novel course, and leads to a product which could scarcely have escaped notice in the present work if it had been present. The absence of this product seems to justify the further conclusion that the cysteine grouping did not take part in thiohydantoin formation, and therefore did not, in the original glutathione, have a free carboxyl group. The further discussion of these results will be given at another time.

The existence of two thiohydantoin rings in the compound, Formula III, is considered to be further independent evidence of the attachment of glutamic acid in glutathione through its $\gamma$-carboxyl group.

## EXPERIMENTAL.

*Glutathione.*—The glutathione used was the reduced form, prepared from yeast. It melted at 191–192° (decomposition) and contained 10.68 per cent S (calculated, 10.42 per cent).

*Condensation of Reduced Glutathione with Ammonium Thiocyanate and Acetic Anhydride.*—Glutathione (0.60 gm.) and ammonium thiocyanate (0.4 gm.) were covered with 4.0 cc. of acetic anhydride containing 10 per cent acetic acid, and heated under an air condenser on a steam bath for 20 minutes. Complete solution took place promptly, and the usual orange color developed. The solution was then cooled, treated with 20 cc. of water, and allowed to stand until the anhydride was hydrolyzed. The aqueous layer was then poured off, and the sticky residue triturated vigorously with 20 cc. of fresh cold water until all stickiness had disappeared. It was then collected on a filter and washed with a little water. The yield was 0.715 gm. (77 per cent) of a product which began to sinter at about 140°, and finally melted indefinitely at about 155° with effervescence. The substance was soluble in alcohol, ethyl acetate, or acetic acid, but could not be obtained in crystalline form when such solutions were allowed to evaporate.

$C_{16}H_{19}O_6N_5S_3$. Calculated. N, 14.80. S, 20.30.
Found. " 14.83 (Kjeldahl). S, 19.85,* 20.02, 19.95.
(Parr bomb).

* The first sample was from another preparation.

*Condensation of the Bis-Thiohydantoin (III) with Benzaldehyde.*—To 0.300 gm. of the bis-thiohydantoin and 0.5 gm. of freshly fused sodium acetate were added 5 cc. of glacial acetic acid and 0.3 cc. of benzaldehyde. The mixture was boiled rather vigorously under a reflux in an oil bath for 1 hour. The solution was then cooled, diluted with 50 cc. of water, and 6 N alkali added until the solution was molar with regard to alkali. After 15 minutes, the solution was acidified with hydrochloric acid, allowed to stand for some time, and filtered. In two experiments, the yields were 65 and 67 mg. of benzal thiohydantoin (calculated, 129 mg.). The product melted at 257°, and a mixture with known pure benzal thiohydantoin melted at the same temperature. The melting point did not change appreciably on recrystallization.

$C_{10}H_8ON_2S$. Calculated. S, 15.68 (Parr bomb).
Found. " 15.55.

Under similar conditions of condensation, pure thiohydantoin gave a 96 per cent yield of the benzal derivative, but when benzoylthiohydantoin was used, not over 75 per cent could be obtained.

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# THIOHYDANTOINS DERIVED FROM CYSTINE AND FROM CYSTEINE.

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The very interesting method developed by Schlack and Kumpf (1) for the step by step degradation of peptides involves the preparation of peptide thiohydantoin derivatives in which, in general, only the amino acid group carrying a terminal carboxyl group takes part in the formation of the hydantoin ring. Very mild alkaline hydrolysis then splits off the thiohydantoin of this particular amino acid, which is thus identified, and its position in the peptide chain demonstrated.

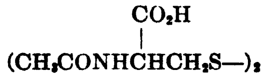
Unfortunately, thiohydantoins derived from cystine and cysteine have not been described, though unsuccessful attempts have been made to prepare them ((1) p. 138). The application of the method of Schlack and Kumpf to peptides containing these amino acids has been thus prevented. As glutathione is such a peptide, the writer was directly interested (2).

The usual method (3) for the preparation of thiohydantoins from amino acids is not directly applicable to cystine. This, however, appears to be due only to the extreme insolubility of cystine in acetic anhydride, which prevents its normal acetylation under such conditions. When cystine is acetylated in alkaline aqueous solution (the isolation of the acetylcystine is not necessary; for its preparation by another and less direct method see Inoue (4)), the residue obtained on evaporation of this solution to dryness (at 10 to 20 mm.) reacts readily with ammonium thiocyanate in acetic anhydride, and thiohydantoin formation takes place. For the structure of compounds discussed in the text see the accompanying formulæ.

From the solution there were isolated (for details, see the experimental part) two solid organic products, and a considerable amount of free sulfur.[1] The less soluble product (IV) melted above 200°, and while appreciably soluble in hot alcohol or glacial acetic acid, dissolved so slightly that it has thus far been purified only by washing with suitable solvents. Among these must be included carbon disulfide, as most of the sulfur liberated in the reaction occurs here, and must be removed. Substance IV is, from its analysis and general behavior, the normally expected product, cystine-bis-1-acetyl-2-thiohydantoin. It was obtained in yields as high as 50 per cent.

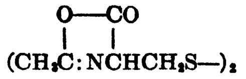
A very much more soluble product, m.p. 142°, practically insoluble in water, but readily soluble in hot alcohol and decidedly soluble even in cold and rather dilute acetic acid, gives analytical values strongly indicating a cysteine-1-S-diacetyl-2-thiohydantoin (V).

Formula VI, which represents an acetylcystine which has undergone thiohydantoin formation on one side only, and which has substantially the same nitrogen and sulfur content as V, is excluded, since the substance actually obtained, when treated further with ammonium thiocyanate and acetic anhydride, gave no IV.



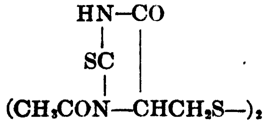
$$(\mathrm{CH_3CONH}\overset{\overset{\mathrm{CO_2H}}{|}}{\mathrm{C}}\mathrm{HCH_2S{-}})_2$$

I.



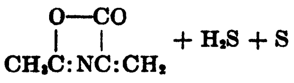
$$(\mathrm{CH_3}\overset{\overset{\mathrm{O{-}}}{|}}{\mathrm{C}}\mathrm{:N}\overset{\overset{\mathrm{CO}}{|}}{\mathrm{C}}\mathrm{HCH_2S{-}})_2$$

II.



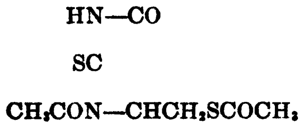
$$(\mathrm{CH_3CO}\overset{\overset{\overset{\mathrm{HN{-}}}{|}}{\mathrm{SC}}}{\overset{|}{\mathrm{N}}}\mathrm{{-}}\overset{\mathrm{CO}}{\overset{|}{\mathrm{C}}}\mathrm{HCH_2S{-}})_2$$

IV.



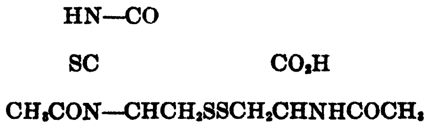
$$\mathrm{CH_3}\overset{\overset{\mathrm{O{-}}}{|}}{\mathrm{C}}\mathrm{:N}\overset{\overset{\mathrm{CO}}{|}}{\mathrm{C}}\mathrm{:CH_2} + \mathrm{H_2S} + \mathrm{S}$$

III.



$$\mathrm{CH_3CO}\overset{\overset{\mathrm{HN{-}}}{\mathrm{SC}}}{\mathrm{N}}\mathrm{{-}}\overset{\mathrm{CO}}{\mathrm{C}}\mathrm{HCH_2SCOCH_3}$$

V.



$$\mathrm{CH_3CO}\overset{\overset{\mathrm{HN{-}}}{\mathrm{SC}}}{\mathrm{N}}\mathrm{{-}}\overset{\mathrm{CO}}{\mathrm{C}}\mathrm{HCH_2SCH_2}\overset{\mathrm{CO_2H}}{\mathrm{C}}\mathrm{HNHCOCH_3}$$

VI.

---

[1] There is a strong presumption that none of this sulfur comes from the thiocyanate, as no free sulfur has been observed in any of the large number of preparations of thiohydantoins from other amino acids.

Moreover, cysteine hydrochloride, submitted to the thiohydantoin condensation (in the presence of a fairly exactly equivalent amount of anhydrous sodium acetate) gave V as the only insoluble product. In this case, no free sulfur was observed.

The remarkable behavior of the two thiohydantoins, IV and V, toward alkali will be the subject of the following paper.

While the formation of a cysteine derivative (V) from cystine requires explanation, the liberation of free sulfur already noted is more surprizing. For an interesting analogy, reference may be made to a series of papers by Bergmann and his associates, and particularly to two by Bergmann and Stather (5), in which it is shown that cystine derivatives in which the $\alpha$-carbon atom is a member of the diketopiperazine ring, readily lose $H_2S_2$ ($H_2S$ and S) when treated with alkali, to form methylene derivatives. Bergmann with others had previously shown that a very similar type of lability was induced in analogous azlactones.

TABLE I.

*Effect of Sodium Acetate.*

| Product. | Portion A. | | Portion B. | |
|---|---|---|---|---|
| | Weight. | Equivalent. | Weight. | Equivalent. |
| | *gm.* | | *gm.* | |
| IV | 1.878 | 0.0101 | 0.007 | 0.0000 |
| V | 0.800 | 0.0034 | 1.163 | 0.0047 |
| S | 0.038 | 0.0024 | 0.205 | 0.0128 |
| IV + S | | 0.0125 (62.5 per cent). | | 0.0128 (64 per cent). |

Now, the first stage in the formation of a thiohydantoin from cystine is probably the azlactone (II) (6). Considering that the formation of this ring has probably made the cystine much more susceptible to the elimination of $H_2S_2$, it requires only the presence of an alkaline reagent to account for the formation of some sulfur, and also of hydrogen sulfide (with the probable incidental formation of III) which, again most effectively in the presence of an alkaline reagent, would be likely to reduce cystine derivatives (*e.g.* II, IV) with the resultant formation of V, and incidentally with the liberation of further amounts of free sulfur. It seems quite certain that the alkaline reagent involved is sodium acetate, which in such a solvent as acetic anhydride is to be regarded as analogous to a strong alkali in water solution.

The actual importance of sodium acetate in determining the proportions in which the two derivatives, IV and V, are formed, and of the amount of sulfur liberated, is shown very definitely in the experimental part of this paper. The results are summarized in Table I. It is to be noted that, according to the preceding discussion, cystine derivatives are destroyed in two ways: (*a*) the elimination of $H_2S$ and S to form methylene derivatives; and (*b*) the reduction by $H_2S$ to cysteine derivatives. Each of these processes should involve the elimination of *1 atom* of sulfur for each *mol* (*2* equivalents) of cystine derivative. Therefore in Table I, $\frac{1}{2}S$ is taken as 1 equivalent, and it should be expected that the sulfur so calculated would be a measure of the cystine derivatives disappearing in the reaction. It is actually found that in the two runs, Experiments A and B (Table I) in which the yield of IV varies from 50 per cent to almost nothing, the sum of IV and the free sulfur (in equivalents) is practically a constant, although the total material is somewhat incompletely accounted for.

## EXPERIMENTAL.

*N-Acetylcystine* (*I*).—Cystine (4.8 gm.) was dissolved in 20 cc. of water to which had been added 9 cc. of 6 N sodium hydroxide (6.7 cc., 1 mol), and 8 cc. of acetic anhydide added at once. The solution was stirred until the anhydride had dissolved, with sufficient cooling to keep the temperature from rising appreciably. An amount of hydrochloric acid just equivalent to the alkali used was then added, and the solution distilled to dryness under a pressure of not more than 30 mm. The dry residue was used directly for further reactions.

*Cystine-Bis-1-Acetyl-2-Thiohydantoin* (*IV*).—In the earlier experiments, it was considered more important to avoid an excess of hydrochloric acid during the evaporation of the acetylcystine solution, than to decompose all of the sodium acetate present. Accordingly, somewhat less acid was used than the amount described in the preceding paragraph. To the dried acetylcystine residue were added 4.0 gm. of ammonium thiocyanate, 18 cc. of acetic anhydride, and 2 cc. of glacial acetic acid, and the mixture was heated under an air condenser on a steam bath for 30 minutes. The cooled residue was then poured into 100 cc. of water, and when the acetic anhydride was completely decomposed, 15 gm. of dry sodium carbonate were slowly added, with continued stirring to avoid any local alkalinity. This was to decrease the solvent action of the acetic acid present. The same results are obtained, somewhat less conveniently, if the acid is largely removed by vacuum distillation.

The sticky material which separated was allowed to stand overnight, and if still sticky was then triturated with a fresh quantity of water until it was no longer so. The mixture was filtered, washed with water, and the filtrate discarded. The material on the filter was washed with 50 cc. of hot alcohol (removal of cysteine derivative (V)) and then with 25 cc. of carbon disulfide. The latter washings, on evaporation, deposited considerable amounts of practically pure sulfur.

The residue on the filter was white and apparently crystalline. It was very slightly soluble in hot alcohol or hot glacial acetic acid, and washing with the latter solvent raised the melting point fairly readily to 208° (effervescence).[2] The yield varied widely, but reached 50 per cent when exactly the calculated amount of hydrochloric acid was used in the preparation of the acetylcystine.

$C_{18}H_{14}O_4N_4S_4$. Calculated. N, 13.80. S, 31.5.
Found. " 14.02 (micro-Kjeldahl). S, 31.4, 31.8 (Parr bomb).

*Cysteine-1-S-Diacetyl-2-Thiohydantoin (V)*.—The alcohol extract from the above preparation was concentrated to 10 cc., cooled, and then judiciously treated with sufficient water to cause the product to separate as a solid. It was frequently necessary to let the solution stand for some time. After recrystallization from dilute alcohol, the product melted at 142°. The best yields of this material (60 per cent) were obtained when the acetylcystine used contained small but appreciable amounts of sodium acetate.

---

[2] A melting point as high as 214–215° (effervescence) was obtained on a sample which was finally digested for some time with hot acetic anhydride. Such variations are accompanied with very little change in the analysis of the products. This high melting sample contained 31.2 per cent sulfur.

$C_8H_{10}O_3N_2S_2$. Calculated. S, 26.0 (Parr bomb).
Found. " 26.41, 26.64.[3]

When the thiohydantoin (V) was again heated with thiocyanate and acetic anhydride, much of the material was recovered unchanged, and there was no evidence of the formation of any of product IV. This result eliminates from consideration Formula VI.

*Preparation from Cysteine.*—Cysteine hydrochloride (1.5 gm.) was condensed with 1.1 gm. of ammonium thiocyanate in 5 cc. of acetic anhydride which contained 0.5 cc. of acetic acid. To liberate the cysteine for reaction, 0.8 gm. (calculated, 0.83 gm.) of anhydrous sodium acetate was added before heating. The only product isolated was the thiohydantoin (V). No free sulfur was observed, and only faint traces of material difficultly soluble in hot alcohol were obtained. The product shown in Formula V obtained, after purification, contained 25.7 per cent sulfur (calculated, 26.0 per cent).

*Influence of Sodium Acetate on the Preparation of IV and V.*—A preparation of acetylcystine from 4.8 gm. of cystine, in which special care was taken to make the added hydrochloric acid just equivalent to the alkali originally used, was divided into two equal portions. One portion (Portion A) was condensed to the thiohydantoins directly, and the other (Portion B) after the addition of 2.0 gm. (0.024 equivalent) of anhydrous sodium acetate. This quantity of acetate was, intentionally, somewhat extreme. The distribution of crude products isolated is shown in Table I.

The difference in the course of the reaction in the two cases is striking. In Portion A, Product IV was obtained in 50 per cent yield, and the amount of sulfur was quite small. In Portion B, over 5 times as much sulfur resulted, and Product IV was practically, if not completely, absent. But the sum (in equivalents; see discussion) of Products IV and S is substantially constant in the two runs. It should be added that Product V obtained from Portion B was very impure, and in fact quite unsuitable for purification. A small fraction of the acetate used in Portion B would give decidedly larger yields of Product V, and a better product.

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[3] This material, from its method of separation, contained a very small amount of free sulfur. When a sample was dissolved in dilute alkali, this remained as an insoluble residue, which had the melting point of sulfur.

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# THE ACTION OF ALKALI ON THIOHYDANTOIN DERIVATIVES OF CYSTINE AND CYSTEINE.

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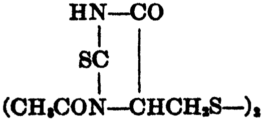
(Received for publication, June 3, 1930.)

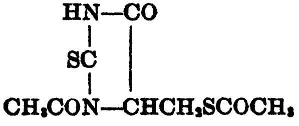
The preparation of cystine-bis-1-acetyl-2-thiohydantoin (Formula I) and cysteine-1-S-diacetyl-2-thiohydantoin (II) has been described in a previous paper (1). Each of these substances dissolves readily in cold dilute alkali, and each, on acidification of these solutions, gives an amorphous, colloidal, white precipitate while the solution has in each case a definite odor of hydrogen sulfide. These precipitates, when collected on a filter and dried, have very similar physical properties. Both are very insoluble in water, and on heating decompose (with much preliminary darkening, but with a final definite effervescence) above 260°. The products are, however, different, as shown by analysis, and it is the purpose of this paper to discuss the reactions involved, and also to call attention to the possible utilization of the products formed for the diagnosis of certain polypeptides.

The product finally obtained from the cysteine derivative (II) after treatment for half an hour with normal alkali at room temperature and acidification with hydrochloric acid, was rather obviously the (polymerized) 2-thio-5-methylenehydantoin (IV). The distinctly low sulfur content (calculated, 25.0 per cent; found, 23.3, 23.8 per cent) is to be attributed to the very gelatinous nature of the precipitates, which makes the complete removal of



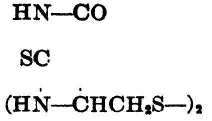
$$\begin{array}{l} \text{HN—CO} \\ \text{SC} \\ (\text{CH}_3\text{CON—CHCH}_2\text{S—})_2 \end{array}$$

I.

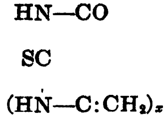


$$\begin{array}{l} \text{HN—CO} \\ \text{SC} \\ \text{CH}_3\text{CON—CHCH}_2\text{SCOCH}_3 \end{array}$$

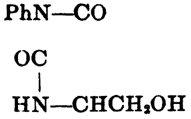
II.



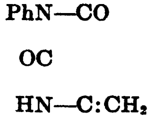
III.



IV.



V.



VI.

salts by washing extremely difficult. Sufficient material for an ash determination was not available.

In the case of the cystine derivative (I) the reaction was decidedly less simple, and it soon became apparent that more than one product was involved. The elimination of the N-acetyl group seems to be practically instantaneous, as in the preceding case; but the subsequent elimination of $H_2S_2$ from (III) is sufficiently slower than that of $CH_3COSH$ in the analogous case, so that a mixture of cystine-bis-thiohydantoin (III) and methylene thiohydantoin (IV) results. This conclusion is based on the sulfur content of the mixture as obtained after alkali treatments of varying severity (for details, see the experimental part), as no method was found for separating the components of the mixture. Various products gave analyses indicating the presence of from 89.5 to 54.2 per cent of (III). Less Product III was regularly found in those cases in which the alkali treatment was more severe.

Rather close analogies exist for the changes which have been described. Reference has been made in the preceding paper to the increased lability of –SH and –OH groups attached to a β-carbon in the side chain of certain cyclic compounds, as explained by Bergmann and his collaborators. In a more recent paper, Bergmann and Delis (2) have shown that the 3-phenylhydantoin of serine (V) is converted by cold dilute alkali to 3-phenyl-5-methylenehydantoin (VI), and even further broken down to phenyl urea and pyruvic acid.[1] The methylene derivative (VI) was

---

[1] In 1911, Wheeler and Hoffman (3) observed the similar formation of anisylpyruvic acid from 5-anisalhydantoin, but in their case warm alkali was necessary.

crystalline, and showed considerable solubility in hot organic solvents, differing in these respects from the methylene derivative (IV) described in the present paper. It showed, however, the same conspicuous insolubility in water, and the same type of behavior (decomposition, without melting, at a high temperature) when heated.[3]

When the hydroxyl group of V was acetylated, the ease of conversion of the resulting derivative to VI by alkali was greatly increased (2). This helps to explain the fact that the acetylated cysteine derivative (II) is very much more rapidly converted to IV than is the cystine derivative (I).

It remains to be stated that, when parallel samples of I and II were treated with cold normal alkali for half an hour and then acidified, the yield of final product obtained was in each case about 65 per cent of the calculated amount. In view of the rather extreme insolubility of the products, the loss is not easily explained except on the basis of some further reaction. In view of the work of Bergmann and Delis (2), it is possible that a splitting to thiourea and pyruvic acid took place, but as this possibility was not suspected at the time, this point was not determined. The ring system here involved is at least moderately stable toward the action of alkali.

Although the two products of the action of alkali on thiohydantoins of cystine and cysteine described in this paper do not lend themselves to recrystallization or to identification by melting point, they are very insoluble, and both have higher sulfur contents than any normal thiohydantoins with the exception of the easily recognized 2-thiohydantoin and 2-thio-5-methylhydantoin. From their physical characteristics and their sulfur contents it is, then, to be expected that they will fit into the procedure of Schlack and Kumpf (5) and permit the extension of their method to the diagnosis of polypeptides containing cystine or cysteine so linked as to present a terminal carboxyl group, and therefore to form a thiohydantoin ring involving these amino acids.

[3] For a discussion of the somewhat unusual relationships of polymeric (or associated) methylene derivatives, see Bergmann and Enszlin (4) and other papers quoted there.

## EXPERIMENTAL.

*Treatment of Cysteine-1-S-Diacetyl-2-Thiohydantoin (II) with Alkali.*—The thiohydantoin (0.5 gm.) was dissolved in 20 cc. of N sodium hydroxide, and allowed to stand (at room temperature) for 30 minutes. The solution was then acidified (the odor of hydrogen sulfide was noticeable), filtered, and the gelatinous precipitate washed. Filtration was very slow, and the washing presumably rather ineffective.

| | | |
|---|---|---|
| $C_4H_4ON_2S$. | Calculated. | S, 25.0 (Parr bomb). |
| | Found. | " 23.8, 23.3. |

The low sulfur content is attributed to adsorbed salts. Unfortunately, not enough of this preparation remained for an ash determination.

*Treatment of Cystine-Bis-1-Acetyl-2-Thiohydantoin (I) with Alkali.*—The substance dissolved readily in an excess of 0.1 N sodium hydroxide. After 5 minutes at room temperature, half the solution was acidified with hydrochloric acid and filtered. There was some odor of hydrogen sulfide from the solution, but the colloidal material which separated was chiefly cystine-bis-2-thiohydantoin (III), as shown by the analysis, which corresponds to that calculated for a mixture of III with 10.5 per cent of IV.

| | | |
|---|---|---|
| $C_8H_{10}O_2N_4S_4$. | Calculated. | S, 39.75 (Parr bomb). |
| | Found. | " 38.2. |

The remaining half of the above solution was then made up to 1.0 N with respect to alkali, and allowed to stand 1.5 hours before acidification. The odor of hydrogen sulfide was stronger, and the product contained 33.0 per cent sulfur, which would indicate the formation of rather more than 50 per cent of the methylene derivative IV in addition to the III present.

Other preparations obtained by the action of N sodium hydroxide for periods of 0.5 to 1 hour, gave analyses showing 36.5, 33.8, and 34.8 per cent sulfur respectively. No solvent other than alkali was found in which any of these preparations were appreciably soluble.

## SUMMARY.

1. The action of alkali on the acetylated thiohydantoins derived from cystine and from cysteine has been described, and the constitution of the products discussed.

2. It is believed that acquaintance with these products will permit the extension of the method of Schlack and Kumpf (5) for the determination of the structure of polypeptides, to peptides containing cysteine.

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# A STUDY OF GLUTATHIONE.

## IV. DETERMINATION OF THE STRUCTURE OF GLUTATHIONE.

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(Received for publication, June 12, 1930.)

In a recent publication (4) it was suggested that the structure of glutathione was either glutamyl cysteinyl glycine or glutamyl glycyl cysteine. The specific reaction which indicated that the glycine was in the terminal position was the formation of a derivative of glycine through the Grignard addition product with the ester of glutathione and phenylmagnesium bromide. It was pointed out that hydrolysis of the tripeptide occurred with great ease in water, even at 38°, and as it seemed possible that the tripeptide was split during the formation of the ester in absolute alcohol in the presence of hydrochloric acid, it became evident that further work concerning the hydrolysis of the tripeptide in alcohol and water was desirable. Accordingly, the ester of glutathione was again prepared and examined for the presence of split-products. If the structure of glutathione were glutamyl glycyl cysteine, the only reaction which could result in the esterification of the carboxyl group of glycine would involve the splitting off of the cysteine during the formation of the ester. The cysteine would be present as its ethyl ester. The ethyl ester of cysteine was found to respond to Sullivan's test (8, 9) with the production of a little more color than an equal weight of cysteine. Some of the ester hydrochloride of glutathione was tested with Sullivan's test, but no trace of the ester of cysteine was found.

The ester hydrochloride of glutathione was then added to phenylmagnesium bromide as already described (4) and the peptide alcohol was separated and fractionated by partial precipitation from alcohol by the addition of ether. The first fraction after drying weighed 300 mg.; the second fraction, 800 mg. These two fractions were then analyzed for the ratio of nitrogen to sulfur. The ratio in the first fraction was 2.6:1; in the second, 2.93:1. The ratio of nitrogen to sulfur indicates that the peptide alcohol was formed between phenylmagnesium bromide and the ester of the tripeptide and is further evidence that significant decomposition of the tripeptide did not occur during the preparation of the ester. The portion with the ratio of nitrogen to sulfur 2.93:1, was then boiled in sodium hydroxide and diphenylethanolamine was separated. This was boiled with acid and the resulting diphenylacetaldehyde converted into diphenylacetaldazine which was separated in a yield of 20 to 22 per cent of the peptide alcohol. This material was identified by its melting point and nitrogen content and by its identity to the same product formed from phenylmagnesium bromide and the ester of glycine.

The observation that the glutamyl grouping was easily split off in distilled water from the other two amino acids suggested that it would be possible to confirm the position of the glycine by a study of the dipeptide obtained after loss of the glutamic acid. The tripeptide was dissolved in distilled water and allowed to stand at 62° for 120 hours. The solution was then treated with mercury sulfate until the maximal precipitation was produced. The precipitate was filtered off and the filtrate analyzed for nitrogen. It was found to contain one-third of the total nitrogen in the tripeptide. The mercury and sulfuric acid were removed from the solution which was evaporated to dryness in a vacuum. The residue was extracted with alcohol. The alcohol-soluble fraction was shown to be pyrrolidone-carboxylic acid. The mercury precipitate was decomposed in water with hydrogen sulfide and various experiments were carried out to show whether the amino group of the cysteine or the amino group of glycine was substituted.

It was first shown that if the glutamic acid is hydrolyzed from the tripeptide in distilled water at 62°, almost no anhydride of glycyl cysteine is formed. The small amount that was formed was oxidized to the disulfide grouping with air in a slightly alkaline solution. The few mg. of the anhydride were removed and the filtrate divided into two portions. One-half was hydrolyzed with hydrochloric acid and the benzoyl derivative of the glycine was then formed. Less than 50 per cent of the theoretical amount was obtained. The other half was treated with excess of sodium hypobromite. The excess of hypobromite was destroyed with hydrogen peroxide, the solution was made acid, concentrated to small volume, hydrolyzed, and the glycine was benzoylated. Hippuric acid was separated; the yield was 19 per cent of the theoretical.

The same type of experiment was carried out after the solution of the tripeptide in distilled water had been heated on the steam bath for 22 hours. As Hopkins (3) has already shown, a larger amount of the anhydride of glycyl cysteine was formed; only 17 per cent of the theoretical amount of glycine could be separated after hydrolysis of the dipeptide which had not formed the dianhydride of glycyl cystine. After a similar solution had been heated for 44 hours at 100° on the steam bath, the amount of anhydride which was separated from the solution by filtration was slightly higher than when the solution was heated for 22 hours, but there was very little glycine which could be recovered after hydrolysis of the filtrate from the anhydride with hydrochloric acid.

Other reagents were also used to determine whether the amino group of the glycine was substituted. The glutamic acid was removed by hydrolysis at 62° and the solution was divided into two equal portions. In one-half the glycine was determined after hydrolysis; the other half was treated with nitrous acid. Hippuric acid was recovered from each half, but again in small yield.

The dipeptide after hydrolysis of the glutamic acid was also treated with 2,3,4-trinitrotoluene as suggested by Barger and applied by Quastel, Stewart, and Tunnicliffe (6) for the determination of free amino groups.

2,3,4-Trinitrotoluene was condensed with the oxidized dipeptide of cystine and glycine. The excess of the nitro compound was removed with benzene and any unchanged peptide with water. The bright yellow condensation product was hydrolyzed with hydrochloric acid. After benzoylation, hippuric acid was recovered in 27 per cent yield. This is evidence that the amino group of the glycine in the dipeptide is substituted. Still further evidence which shows that the amino group of the cysteine is free was given by separation of a fraction of the condensation product of the dipeptide and trinitrotoluene after hydrolysis with hydrochloric acid. This was separated in crystalline form and found to contain sulfur and nitrogen in amounts corresponding to those in the dinitrotoluene derivative of cystine. This is evidence that the amino group of the cysteine in the dipeptide is not substituted.

The reactions of the dipeptide, after separation of the glutamyl grouping, with sodium hypobromite, nitrous acid, and trinitrotoluene all indicate that the amino group of the glycine is substituted. These results confirm the conclusion based on the reaction of the ester of the tripeptide with the Grignard reagent and clearly show that the glycine is in the terminal position.

The glycyl grouping in the dipeptide appears to be easily destroyed. The yields of benzoyl glycine which were recovered after treatment of the dipeptide with hypobromite, nitrous acid, trinitrotoluene, and even after heating in water are all far below the theoretical amounts. The results on the dipeptide obtained after separation of the glutamyl grouping indicated that the sulfhydryl group influences the course of the reaction with the various reagents. When glutathione was first treated with sodium hypobromite, it was in the form of the sulfonic acid derivative which was prepared by the action of bromine in normal hydrochloric acid. It was therefore decided to repeat these experiments on glutathione with the sulfur present in the sulfhydryl form.

In the experiments already published (4), with the sulfonic acid derivative of glutathione, it was possible to separate glycine in good yield after treatment with sodium hypobromite. Furthermore, succinic acid derived from the glutamyl grouping was separated in yields as high as 50 per cent of the theoretical amount. When glutathione with its sulfur in the form of the sulfhydryl group is treated with sodium hypobromite, only a small percentage of the total glycine can be recovered after acid hydrolysis and either no succinic or only a small amount of succinic acid was formed. There was evidence, however, of the formation of $\alpha$-hydroxyglutaric acid. It was furthermore found that the results were greatly affected by the order of the addition. If the solution of glutathione was added to the solution of sodium hypobromite, more glycine and less succinic acid were recovered than if the hypobromite was slowly added to a solution of glutathione. Another important factor which may explain these results is the alkalinity of the solution. Goldschmidt, Wilberg, Nagel, and Martin (2) have shown that amino acids are converted into nitriles only in an alkaline solution.

In the previously described experiments, the action of hydrogen peroxide on the ammonium salt of glutathione was shown to destroy the glycyl grouping, but hydrogen peroxide oxidized the glutamic acid to succinic acid in a yield of 20 per cent. The action of hydrogen peroxide has now been studied on the ammonium salt of glutathione after treatment with bromine in an acid solution, and it has been found that if the sulfur is thus oxidized to a sulfonic acid derivative, glycine can be recovered after treatment with hydrogen peroxide and a much higher yield of succinic acid is formed from the glutamyl grouping. It therefore becomes evident that the sulfhydryl group has a marked influence on the course of oxidation with sodium hypobromite and with hydrogen peroxide.

The results published in this paper confirm those which we have previously obtained. They firmly establish the structure of glutathione to be glutamyl cysteinyl glycine. The recent work of Nicolet (5) also confirms this structure of the tripeptide.

## EXPERIMENTAL.

*Glutathione Ester. Examination for Possible Presence of Cysteine Ester.*—The ester hydrochloride was prepared from 3 gm. of glutathione as previously described (4). 50 mg. of the dry material were dissolved in 100 cc. of 0.1 N hydrochloric acid. Varying amounts up to 5 cc. of this solution were used for Sullivan's test (8, 9). No trace of the color characteristic of cysteine was obtained, although cysteine ethyl ester was found to yield even more color than an equal weight of cysteine. Since as little as 0.2 mg. of cysteine ester hydrochloride could be easily detected, there could not be more than 8 per cent of cysteine ester hydrochloride in the product.

*Fractionation of the Peptide Alcohol from the Grignard Reaction Product of Glutathione Ester.*—Although Bettzieche (1) and coworkers have not found any instance of addition of the Grignard reagent to a -CONH- group, such a possibility should be excluded. If glutathione were glutamyl glycyl cysteine, addition of the Grignard reagent to the carbonyl group of the glycyl grouping as well as to the ester groups might conceivably account for the formation of the diphenylethanolamine previously reported. Such a reaction would likewise produce the amino alcohol derived from cysteine. It was found that the product of the action of the Grignard reagent on cysteine ethyl ester hydrochloride is almost completely precipitated from its alcoholic solution by the addition of 5 volumes of ether. The product so obtained from the ester of glutathione is not so completely precipitated in this way. Consequently, fractional precipitation of the latter product should concentrate any derivative of cysteine in the first fraction.

The crude peptide alcohol hydrochloride was prepared as already described. It was dissolved in alcohol and the free base precipitated with ammonia. After dilution with water the mixture was extracted with ether. The extract was dried with sodium sulfate and the ether and alcohol removed under reduced pressure. The residue was dissolved in a minimal amount of absolute alcohol and 5 volumes of ether were added. After an hour the precipitate was separated and dried. Weight 0.31 gm. The filtrate was evaporated to a small volume and again diluted with ether. Very little remained in solution. The second fraction weighed 0.80 gm.

*First Fraction Analysis.*—Substance 11.4 mg., 11.4 mg.; N (micro-Kjeldahl) 0.784 mg., 0.781 mg. Substance 0.1008 gm.; $BaSO_4$, 0.0445 gm. Calculated for $C_{34}H_{37}O_4N_3S$. N, 7.20; S, 5.49. Found. N, 6.88; S, 6.07. N:S = 2.6:1.

*Second Fraction Analysis.*—Substance 11.02 mg., 11.02 mg.; N (micro-Kjeldahl) 0.839, 0.835. Substance 0.1791 gm.; $BaSO_4$, 0.0774 gm. Calculated for $C_{34}H_{37}O_4N_3S$. N, 7.20; S, 5.49. Found. N, 7.62, 7.57; S, 5.94. N:S = 2.93:1.

The high values of both nitrogen and sulfur indicate incomplete phenylation of the glutathione ester.

*Hydrolysis of Peptide Alcohol and Formation of Diphenylacetaldazine.*—0.60 gm. of the peptide alcohol with the ratio of N:S 2.93 was refluxed 6 hours in 60 cc. of 40 per cent alcohol containing 5 per cent of sodium hydroxide. The cold solution was extracted thoroughly with ether. The ether was extracted with 10 per cent hydrochloric acid. The acid extract (50 cc.) was diluted with 300 cc. of 10 per cent hydrochloric acid and distilled. The distillate was extracted five times with ether. The ether, after drying with sodium sulfate, was distilled. The residue was extracted with three 5 cc. portions of alcohol. The alcohol solution was filtered and to it was added 0.3 gm. of hydrazine sulfate in 35 cc. of water. The mixture was shaken for ½ hour. The precipitate was separated and dried. The diphenylacetaldazine thus obtained weighed 0.038 gm. or 19 per cent of theory. It melted at 170–171°.

*Analysis.*—Substance 9.2 mg., 9.1 mg.; N (micro-Kjeldahl) 0.645 mg., 0.640 mg. Calculated for $C_{28}H_{24}N_2$. N, 7.22. Found. N, 7.01, 7.03.

The alkaline hydrolysis gives much better yield of diphenylacetaldehyde than the acid hydrolysis previously described.

*Action of Sodium Hypobromite on Partially Hydrolyzed Glutathione.*—3.1 gm. of glutathione were dissolved in 50 cc. of water and heated at 62° for 120 hours. The solution in the flask was covered with a small amount of toluene. Mercury sulfate was added to the cooled solution until no more precipitate formed. The mercury precipitate was removed by filtration, and decomposed with hydrogen sulfide. The filtrate from the mercury precipitate contained 97 cc. of 0.1 N nitrogen. The solution of the decomposed mercury precipitate contained 184 cc. of 0.1 N nitrogen. Sulfuric acid was removed from the solution of the mercury precipitate and barium hydroxide was added until phenolsulfonephthalein indicated neutrality. Air was passed through the solution until all the SH had been oxidized by SS. The barium was then exactly removed with sulfuric acid and the solution was concentrated to 30 cc. in a vacuum.

The solution was allowed to stand 18 hours. 15 mg. of the dianhydride of glycyl cystine were removed by filtration. The solution was then divided into two equal parts. A half was hydrolyzed with constant boiling hydrochloric acid; the solution was benzoylated and the hippuric acid separated. The hippuric acid weighed 0.405 gm. This is 45 per cent of the theoretical amount.

The other half was treated with an excess of sodium hypobromite (2) at 0°. The alkalinity of the solution was 0.1 N. The excess hypobromite was destroyed with hydrogen peroxide. The solution was made acid, concentrated to small volume, hydrolyzed with hydrochloric acid, and was benzoylated. The hippuric acid which separated slowly from solution weighed 0.175 gm. This is 19.5 per cent of the theoretical amount.

*Partial Hydrolysis of Glutathione at 100°.*—3 gm. of glutathione were dissolved in 60 cc. of distilled water and heated at 100° for 22 hours. The solution was cooled and mercury sulfate was added until no more precipitate formed. After decomposition of the mercury precipitate with hydrogen sulfide, the solution was made neutral to phenolsulfonephthalein and air was passed through the solution for 18 hours. It was then allowed to stand for 24 hours at 0°. 300 mg. of the dianhydride of glycyl cystine were filtered from the solution. The filtrate from the dianhydride of glycyl cystine was hydrolyzed with hydrochloric acid and the solution benzoylated. Only 0.150 gm. of hippuric acid was recovered. This is 17 per cent of the theoretical amount.

In another experiment which was carried out in the same manner, the solution was heated for 44 hours. 410 mg. of the dianhydride of glycyl cystine were filtered from the solution. Hippuric acid could not be separated from the filtrate of the dianhydride after hydrolysis with hydrochloric acid and benzoylation.

The nitrogen in the three solutions obtained by removal of the mercury precipitate from the partially hydrolyzed glutathione was shown to be present in the form of pyrrolidonecarboxylic acid and not glutamic acid. These results will be reported in another communication.

*Hydrolysis of Dipeptide.*—0.5 gm. of the material which as obtained from glutathione by loss of the glutamyl grouping at 62° and which subsequently had been oxidized to the disulfide form with potassium ferricyanide was hydrolyzed with 45 cc. of 20 per cent hydrochloric acid for 16 hours. The hydrochloric acid was removed by evaporation to dryness under reduced pressure. The residue was dissolved in 10 to 12 cc. of water. This solution was made slightly alkaline with ammonia. After standing overnight 0.25 gm. of cystine (74 per cent of theory) was obtained. The filtrate was treated with benzoyl chloride and sodium hydroxide as previously described. 0.37 gm. of hippuric acid was obtained. This contained 74 per cent of the glycine in 0.5 gm. of the oxidized dipeptide.

Other experiments on the hydrolysis of the dipeptide in which the cystine and glycine were separated by precipitation of the former with mercuric sulfate resulted in much lower yields of hippuric acid and also of cystine. The yields of hippuric acid varied from 30 to 43 per cent. The lower yields may be accounted for by destruction of the amino acids and by adsorption on the mercuric sulfide and barium sulfate precipitates.

*Effect of Nitrous Acid on the Dipeptide of Cysteine and Glycine.*—3.1 gm. of glutathione were dissolved in 100 cc. of water in a small flask. The temperature of the solution was maintained at 62°

for 120 hours. The solution was cooled and treated with mercury sulfate. The precipitate was filtered off. The filtrate from the mercury sulfate contained 100 cc. of 0.1 N nitrogen. Practically all of this was shown to be in the form of pyrrolidonecarboxylic acid. The mercury precipitate was suspended in water and decomposed with hydrogen sulfide. This solution contained 189 cc. of 0.1 N nitrogen. The solution was divided into two parts. One-half was hydrolyzed and the glycine determined after hydrolysis. A quantitative result could not be obtained in this experiment due to an accident. Hydrochloric acid was added to the other half until the solution was normal in respect to this acid. It was then cooled to 5° and the sulfhydryl group was oxidized with bromine. 2 gm. of sodium nitrite were added and the solution was allowed to remain at room temperature for 20 minutes. The nitrous acid was removed and the solution was concentrated to small volume in a vacuum. The solution was benzoylated. 0.085 gm. of hippuric acid were separated. This is 9.5 per cent of the theoretical amount.

*Condensation of the Dipeptide of Cysteine and Glycine with 2,3,4-Trinitrotoluene.*—Some of the dipeptide which was obtained from glutathione through loss of the glutamyl grouping at 62° was oxidized with potassium ferricyanide in a solution made slightly alkaline with barium hydroxide. The solution was acidified with sulfuric acid and sufficient ferric and ferrous sulfates added to remove the ferro- and ferricyanides present. The filtrate was evaporated to a small volume and alcohol added until it reached a concentration of 80 per cent. The potassium sulfate was filtered off. Most of the alcohol was evaporated under reduced pressure, water was added to make a volume of 200 cc., and the sulfuric acid exactly removed with barium hydroxide. The filtrate was evaporated to 5 cc. and poured into 200 cc. of absolute alcohol. The precipitate was filtered off after a few hours.

0.85 gm. of this product was dissolved in 10 cc. of water. To this were added 1.5 gm. of 2,3,4-trinitrotoluene (6) dissolved in 40 cc. of absolute alcohol. The suspension was heated in a boiling water bath for 3 hours. The liquid was then poured off and the gummy substance which adhered to the bottom of the flask was dissolved in 5 cc. of water. The original liquid was then poured back together with 15 cc. of alcohol. The flask was heated for 2 hours longer: the alcohol was bright yellow in color. The contents of the flask were evaporated to dryness. The solid material was extracted once with 10 cc. of water to remove any unchanged dipeptide. Alcohol and water were removed in a vacuum. Unchanged trinitrotoluene was removed from the dry material by three extractions with hot benzene. The residue, a bright yellow solid, weighed 1.20 gm. The trinitrotoluene recovered weighed 0.80 gm. The amount which reacted (0.70 gm.) corresponds to 1.10 gm. of the oxidized dipeptide-trinitrotoluene condensation product. The condensation product is somewhat soluble in water, soluble in alcohol, ethyl acetate, and acetone. It is soluble in a solution of sodium carbonate from which it is precipitated as a gum by acids.

This product was boiled 9 hours with 150 cc. of 20 per cent hydrochloric acid. The cold solution was extracted with ether until no further color was removed. To this extract was added the ether solution of some gummy material which adhered to the walls of the flask. The ether solution was extracted three times with a 2 per cent solution of sodium carbonate. The sodium carbonate solution was washed once with ether, acidified strongly with concentrated hydrochloric acid, heated to boiling, and allowed to stand 18 hours. 0.220 gm. of brownish yellow crystals separated from the solution. A portion was recrystallized by solution in sodium carbonate solution and precipitation with hydrochloric acid. The melting point of the crystals was 190–195°.

*Analysis.*—Substance 10.1, 9.8 mg.; N (micro-Kjeldahl, as modified by Stewart (7)) 1.448, 1.410 mg. Substance 0.0722 gm; $BaSO_4$, 0.0530 gm. Calculated for $C_{20}H_{20}O_{12}N_6S_2$. N, 14.0; S, 10.67. Found. N, 14.3, 14.4; S, 10.1.

An attempt was made to condense cystine and 2,3,4-trinitrotoluene but without success. An attempt was then made to use the dimethyl ester of cystine. Apparently reaction occurred but the product after hydrolysis could not be crystallized as described above. It was soluble in sodium carbonate solution but precipitated as a gum on the addition of acid.

*Separation of Glycine as Hippuric Acid.*—The acid solution which remained after extraction with ether was evaporated to dryness in a vacuum. The residue was heated to boiling with 0.5 cc. of concentrated hydrochloric acid and 25 cc. of absolute alcohol. The solution was filtered, cooled, and diluted to 600 cc. with ether. A white crystalline precipitate separated on standing. This was filtered and the filtrate evaporated to about 10 cc. Upon addition of ether, more of the crystals were obtained. The total mount was 0.48 gm. It was dissolved in water and mercuric sulfate added until there was no further precipitation. The small precipitate was centrifuged and washed with water. It was decomposed with hydrogen sulfide. The hydrogen sulfide was removed by aeration and the sulfuric acid with barium hydroxide. One drop of ammonia was added to the final filtrate and air passed through the solution until the nitroprusside reaction was negative. It was then evaporated to 4 cc. and set aside. Cystine separated in the typical hexagonal plates. Weight, 0.023 gm. This is only 6 per cent of the cystine present in the dipeptide taken.

The filtrate from the precipitate obtained with mercuric sulfate was treated with hydrogen sulfide to remove the mercury. The hydrogen sulfide was removed by aeration and the sulfuric acid with barium hydroxide. The final filtrate was evaporated to 25 cc. Benzoyl chloride (5 cc.) and an excess of 5 N sodium hydroxide were added. The mixture was shaken until the odor of benzoyl chloride disappeared. It was then acidified with hydrochloric acid and thoroughly extracted with ethyl acetate. The ethyl acetate was dried and distilled. The residue was dissolved in chloroform and allowed to stand in the ice box overnight. 0.150 gm. of hippuric acid (melting point 183°) was thus obtained. This was 27 per cent of the theoretical amount derivable from the weight of condensation product used.

*Action of Sodium Hypobromite on Glutathione in the SH Form.*—3.1 gm. of glutathione were dissolved in water and cooled to 0°. 400 cc. of 0.4 N sodium hypobromite were cooled to 0° and added to the glutathione (2). The hypobromite solution contained an excess of sodium hydroxide which made the final concentration 0.1 N in respect to sodium hydroxide. 1100 cc. of 0.1 N sodium hypobromite were reduced. The excess hypobromite was destroyed with hydrogen peroxide. The solution was acidified with hydrochloric acid and concentrated to small volume. It was then hydrolyzed with constant boiling hydrochloric acid, the hydrochloric acid was distilled off until the volume was about 20 cc., the residue was then extracted with ether in a constant extraction apparatus. The ether solution was found to contain only traces of succinic acid but an oil which was later identified as butyrolactone and a halogen-containing organic acid which undoubtedly was α-chloroglutaric acid, were shown to be present. The solution after extraction with ether was treated with benzoyl chloride and 0.590 gm. of hippuric acid was separated. This is 33 per cent of the theoretical amount.

In two of the experiments the conditions were changed. The glutathione was dissolved in 2 equivalents of sodium hydroxide. In one experiment the neutral solution of glutathione was added at 0° to 400 cc. of a solution that contained 1600 cc. of 0.1 N sodium hypobromite and sodium hydroxide sufficient to make the solution 0.1 N with respect to this alkali. The reduction of the hypobromite was the same as before, approximately 1100 cc. of 0.1 N. Only traces of succinic acid were found in the ether extract of the solution after it had been hydrolyzed in 8 per cent sodium hydroxide and had been acidified with hydrochloric acid. When the ether-soluble material was distilled from a water solution after the ether had been removed, an oil passed over with the distillate. This oil was neutral and was shown to be a lactone by its neutralization of sodium hydroxide when an aliquot portion was boiled with a few cc. of 0.1 N alkali. That the oil was butyrolactone which had been formed from the glutamyl grouping was shown by its oxidation to succinic acid. The oil was treated with barium hydroxide and was then evaporated to dryness. To this was added fuming nitric acid and the nitric acid was then removed by evaporation to dryness. The residue was dissolved in water and made alkaline. Barium succinate which formed was removed by filtration and was decomposed by just the required amount of sulfuric acid. Crystals of succinic acid were separated which weighed 0.110 gm. From the solution which had been extracted with ether 0.660 gm. of hippuric acid were recovered after benzoylation.

The same weight of glutathione, 3.1 gm., was neutralized and cooled to 0° and to this solution was then added the same amount of sodium hypobromite which was used in the previous experiment.

All of the volumes, concentration of alkali, and temperatures were the same. The excess of hypobromite was destroyed with hydrogen peroxide and sodium sulfite and the solution was concentrated and hydrolyzed by boiling for 6 hours with 8 per cent sodium hydroxide. It was made acid with hydrochloric acid and extracted with ether. The ether contained 0.360 gm. of succinic acid which was separated in crystalline form from the barium salt. The filtrate from the barium succinate was oxidized with fuming nitric acid and the succinic acid which was formed was recovered through its barium salt. This was decomposed with just sufficient sulfuric acid. The succinic acid weighed 0.105 gm. This result also indicates that the hypobromite had converted the glutamyl grouping into hydroxy or ketoglutaric acid instead of into carbon dioxide and cyanopropionic acid. From the solution after extraction with ether, 0.375 gm. of hippuric acid (21 per cent of theory), was recovered after benzoylation.

*Oxidation of Glutathione in the Form of Its Sulfonic Acid Derivative with Hydrogen Peroxide.*—3.1 gm. of glutathione were dissolved in 50 cc. of N hydrochloric acid and cooled to 0°. Bromine was added to the solution until a distinctly yellow color remained after the addition of a small drop of bromine. After the bromine was completely reduced, the hydrochloric and hydrobromic acids present were neutralized with sodium hydroxide. The solution was then made neutral with ammonia and made to contain 5 per cent hydrogen peroxide. The solution was heated at 70° for 4 hours. The excess of hydrogen peroxide was decomposed with manganese dioxide. The solution was concentrated to 50 cc. and hydrolyzed 6 hours with constant boiling hydrochloric acid. It was evaporated to small volume and extracted with ether in a constant extraction apparatus. The ether was distilled off and the aqueous solution of the residue was treated with barium hydroxide. Barium succinate was filtered off and decomposed with just sufficient sulfuric acid. The succinic acid weighed 0.525 gm. This is 44 per cent of the theoretical amount. The solution which had been extracted with ether was benzoylated. 0.435 gm. of hippuric acid was separated. This is 24 per cent of the theoretical amount. In a previous paper (4) we have shown that when glutathione with the sulfur in the sulfhydryl form is treated with hydrogen peroxide the yield of succinic acid was 20 per cent of theoretical: glycine could not be recovered in the form of hippuric acid.

## SUMMARY.

Cysteine is not split off from glutathione during the preparation of the ester of the tripeptide in absolute alcohol with hydrochloric acid gas. The ester hydrochloride of glutathione will react with phenylmagnesium bromide and form the Grignard addition product in which the ratio of nitrogen to sulfur is 3:1. From the Grignard addition product, diphenylacetaldehyde can be separated. This is evidence that the carboxyl group of the glycine in glutathione is not substituted.

In a water solution at 62° the glutamyl grouping of glutathione is completely hydrolyzed from the dipeptide of cysteine and glycine. Only traces of the anhydride of glycyl cysteine are formed under these conditions.

The dipeptide of cysteine and glycine prepared by partial hydrolysis of glutathione with loss of the glutamyl grouping was treated with sodium hypobromite, nitrous acid, and trinitrotoluene. These reactions show that the amino group of the cysteine is free and that the amino group of the glycine is substituted. The structure of glutathione is glutamyl cysteinyl glycine.

Hydrogen peroxide converts the glutamyl grouping of glutathione into succinic acid with a yield of about 20 per cent when the sulfur is in the sulfhydryl form. Glycine cannot be recovered after treatment with hydrogen peroxide. When glutathione is in the form of its sulfonic acid derivative, hydrogen peroxide will convert a much higher percentage of the glutamyl grouping into succinic acid and a large amount of the glycine may be recovered. The sulfhydryl group exerts a marked influence on the course of the oxidation with hydrogen peroxide.

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# NOTE ON A PREVIOUSLY UNRECORDED OCCURRENCE OF CRYSTALLINE GLOBULIN IN BANANA SEEDS.

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This note records the occurrence of crystalline globulin in banana seeds. In the course of the examination of some banana seeds obtained from the United Fruit Company, it was observed that the starchy endosperm contained large, well developed crystals of the octahedral type. These were found to be particularly well formed and especially numerous in the seed of the Bola variety, although they were also found in the following varieties; Martini, Seminifera, Alis, Kacoloon, Bastard Hemp, Belox, and Zampa.

The crystals, as already indicated, are located in the cells of the endosperm, usually one crystal in a cell, and embedded in, or surrounded by, a compact mass of very small starch grains, these starch masses having the shape of the cell in which they are located (Fig. 1). In size the crystals were found to vary from $15\mu$ to $50\mu$ with an average of $32\mu$. Their occurrence *in situ* in banana seeds appears not to have been previously recorded, in so far as a search of the literature has revealed, and therefore it has seemed worth while to call attention to their presence. Jones and Gersdorff (1, 2) previously have described the isolation of crystalline globulins from the squash, cantaloupe, and sesame seeds. Their work showed no points of difference in the globulins from the cantaloupe and squash seeds in so far as their chemical and physical properties were concerned (1). Studies of the globulin from banana seeds have shown that its optical and microchemical properties are identical with those of the globulin obtained from the squash and cantaloupe seeds. The regular octahedra can be readily examined by powdering the starchy endosperm and mounting in a suitable menstruum. These octahedra (Fig. 2.), like those of the squash and cantaloupe seeds, were found to have an $n$ = approximately 1.545. Their identity was also confirmed by the usual microchemical tests for proteins.

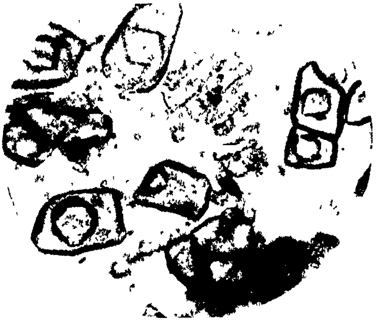

Fig. 1. Globulin embedded in starch masses (×126).

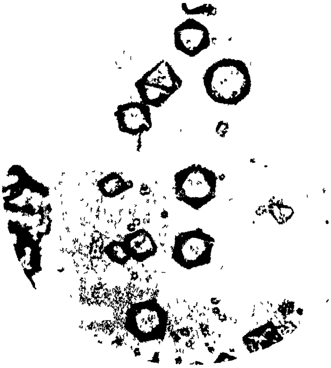

Fig. 2. Crystalline globulin from banana seed (×126).

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# A CRYSTALLINE DERIVATIVE OF AN ACID PRESENT IN LIVER, ACTIVE IN PERNICIOUS ANEMIA.

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The writers have already reported (1) the isolation from liver of a product possessing marked acidic properties, which was highly active, as judged by the reticulocyte response, after administration to suitable cases of pernicious anemia. The present preliminary communication deals with a finely crystalline salt derived from the above product, which has retained its biological potency after three recrystallizations, and also the identification of β-hydroxyglutamic acid among the products of hydrolysis of the active fraction. Several communications on the material in liver active in pernicious anemia have been published by Cohn (2) and his collaborators and by the authors.

The crude material from which we have prepared the crystalline salt was obtained by a combination of procedures which our previous work had shown to yield active products and may be summarized as follows: A strong aqueous solution of commercial liver extract (Lilly) was saturated with picric acid and then repeatedly extracted with a mixture of equal parts of butyl alcohol and ether. The active material is not extracted, while most of the ordinary bases and amino acids are removed. The aqueous residue, after removal of most of the picric acid and solvents, is treated with excess of freshly precipitated lead hydroxide. The active material remains in solution. A further precipitate is obtained with lead acetate and either barium hydroxide or ammonia. This precipitate contains much hypoxanthine, mainly combined with pentose, and after regeneration is clinically inactive. The filtrate, after removal of lead and barium with a very slight excess of sulfuric acid, is precipitated with phosphotungstic acid in the presence of 3 per cent sulfuric acid. The active material is largely precipitated and is recovered by careful decomposition of the precipitate in acetone solution with barium hydroxide. After concentration of the slightly acid solution at low temperatures the active material is treated with an excess of barium hydroxide and at once precipitated with a large excess of absolute alcohol. The barium salt on quantitative decomposition gives a product insoluble in absolute alcohol, but readily soluble in water and dilute alcohol, which is highly potent when tested clinically. The amorphous material is strongly acid to litmus and methyl red, contains approximately 46.6 per cent carbon, 6.9 per cent hydrogen, and 10.6 per cent nitrogen. Free amino nitrogen is absent, but after acid hydrolysis approximately half of the nitrogen is obtained in that form. The diazo and Molisch reactions are negative, permanganate reduction negative in acid and slight in alkaline solution, and apart from phosphotungstic acid and phosphomolybdic acids no precipitates are obtained with the usual base reagents such as picric acid, flavianic acid, gold chloride, etc. On heating with soda-lime the vapors give an intense pyrrole reaction. With β-naphthol and sulfuric acid a strong, green fluorescent solution results resembling that obtained with β-hydroxyglutamic acid. After hydrolysis of the crude product with boiling 10 per cent sulfuric acid, we have found pyrimidine bases absent and have sought unsuccessfully for most of the common amino acids and bases. We have however identified β-hydroxyglutamic acid and have obtained evidence of the presence of a neutral substance precipitated by phosphotungstic acid which awaits complete identification. The β-hydroxyglutamic acid was isolated as silver salt as described by Dakin (3, 4) and then converted into the free acid with hydrogen sulfide. The properties of the substance are identical with those previously recorded.

*Analysis.* $C_5H_9O_5N$.
Calculated. C 36.8, H 5.52, N 8.59.
Found. " 36.9, " 5.45, " 8.97 (Dumas).
" 8.0 (Van Slyke).

The substance gave a typical reaction with β-naphthol, a diazo test in the presence of hot sodium hydroxide, a pyrrole reaction, etc. The strychnine salt was prepared and after recrystallization from moist butyl alcohol had the properties already recorded, while on oxidation with chloramine-T and subsequent treatment with nitrophenylhydrazine, the highly characteristic osazone melting at 297° was obtained as described by Dakin for the acid of protein origin.[1]

The acidic properties of the clinically active material naturally indicated the possibility of purification by means of salts.

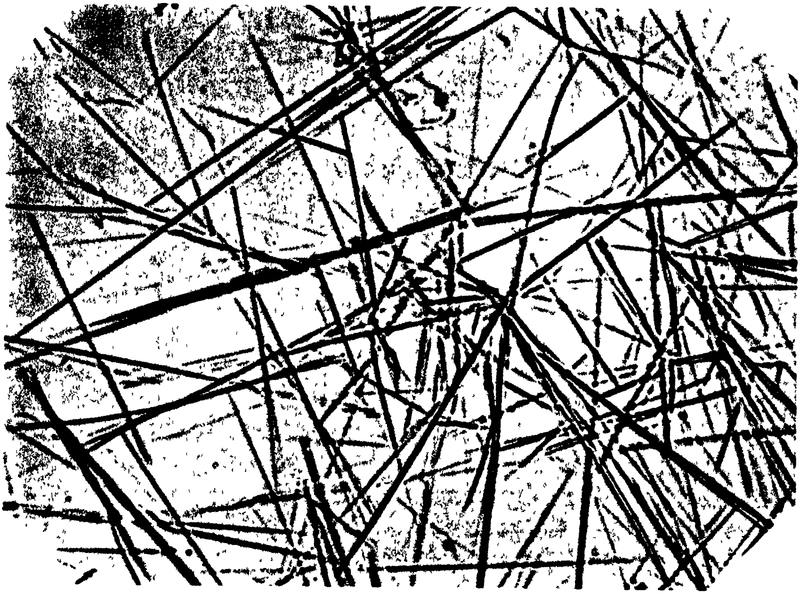

Fig. 1. Twice crystallized quinine salt. × 400 approximately.

Metallic salts soluble in water but precipitable by alcohol, *e.g.* barium, copper, etc., were prepared but could not be crystallized.

A variety of alkaloids was next investigated. Strychnine gave a crystalline salt which was difficult to purify while the brucine, cinchonine, and cinchonidine salts are still less satisfactory. Qui-

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[1] It may perhaps be worth noting in this connection that Dakin ( (4) p. 426) wrote in 1919, "The reversibility of many biological reactions lends significance to the extraordinarily easy conversion of β-hydroxyglutamic acid into hydroxypyrrolidonecarboxylic acid. . . . Such changes may well be concerned with the synthesis of substances containing the pyrrole nucleus. . . . "

nine however gave a finely crystalline salt which could be readily recrystallized. The salt was prepared by gently warming a solution of the acid and adding quinine by degrees until the reaction was neutral or feebly alkaline to litmus. After being filtered from any undissolved base the solution was concentrated at low temperature and allowed to crystallize. The crystals were filtered off and recrystallized from water. The salt separated in the form of long glistening needles as shown in Fig. 1. The crystals, after drying in the desiccator, contain no water of crystallization, melt at 118°, and while fully soluble in cold water are extremely soluble in hot water.

5.280 mg. substance: 12.020 mg. $CO_2$ and 3.43 mg. $H_2O$ = 62.1 per cent C and 7.1 per cent H.

4.593 mg. substance: 0.385 cc. N at 28° and 753 mm. = 9.41 per cent N.

The above results approximate those required for a salt of the formula $C_{20}H_{24}N_2O_2 \cdot C_{10}H_{18}N_2O_6$, but this is naturally subject to revision.

The specific rotation of the salt in aqueous solution was found to be −97°. On decomposing the quinine salt with soda and removing the quinine (55 per cent) a solution was obtained which after hydrolysis contained amino nitrogen and gave the qualitative reactions for β-hydroxyglutamic acid. Some evidence has been obtained of the presence of a salt of higher quinine content.

The activity of the crystals has been tested on three patients with pernicious anemia. Case I, with red blood cells at 1.7 millions, received 310 mg. (after removing quinine) of once crystallized quinine salt intravenously. The reticulocytes rose from 3 to 30 per cent. Case II, with red blood cells at 2.4 millions, received 340 mg. (including quinine) of twice crystallized quinine salt intravenously. The reticulocytes rose from 1 to 16 per cent. Case III, with red blood cells at 1.1 millions, received 70 mg. (excluding quinine) of thrice crystallized quinine salt intravenously. The reticulocytes rose from 0.9 to 10.7 per cent.

The significance of the preceding observations on the structure of the active principle in liver is sufficiently obvious not to require further emphasis at this stage of the investigation. Naturally the possibility of some adsorbed trace of highly active hormone cannot be entirely excluded at present.

We wish to thank the Committee on Pernicious Anemia of the Harvard Medical School for their courtesy in enabling us to obtain liver extract from the Eli Lilly Company to whom we are also indebted. We have enjoyed the indispensable advice and guidance of Dr. H. D. Dakin in this work.

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# THE NORMAL PIGMENT OF THE URINE.

## III. A NEW METHOD FOR ITS EXTRACTION.*

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### INTRODUCTION.

The study of the excretion of the normal urinary pigment, urochrome, under varied experimental conditions (1) and in a number of pathological individuals (1, 2) has permitted the conclusion that the pigment is an endogenous product which is eliminated in quantities proportional to the metabolism. The genesis of so called urochrome and its precise chemical nature still remain to be disclosed. The development of a reliable technique for its isolation appears to be the logical first step in the solution of the above and related problems. It is the purpose of this communication to describe a new method, employed during the past 3 years with apparent effectiveness, for the extraction of the pigment from large volumes of urine.

A rather voluminous literature upon the methods for isolating the normal pigment of the urine has accumulated since the original contribution upon this subject in 1800. Space does not permit a critical review in an article of this type and the reader is referred to the original papers (3–19).

Personal experience with the older methods (12, 13, 16, 18, 19) has indicated that certain objectionable features are present in each. The procedures which have been used and which should be avoided are: (*a*) concentration of the urine by boiling (3–5, 11), (*b*) use of mineral acids (11, 19), (*c*) use of metallic precipitants (7–12).

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* A preliminary report of part of this work has appeared in the Proceedings of the American Society of Biological Chemists (Drabkin, D. L., *J. Biol. Chem.*, **74**, p. xv (1927)).

Concentration of the urine by boiling yields final products which are resinous in nature. Concentration before a process such as extraction may also increase the possibility of contaminating the urinary pigment with substances such as the "humin bodies," monoamino acids, arginine, and histidine (20–23), with which it is closely associated in the urine.

The use of mineral acid brings about progressive changes in composition of the urinary pigment, which may be indicated merely by changes in solubility (19), by the resinous character of the final product, or the recovery of melanin-like substances (evidence of advanced decomposition).

The precipitation of the normal urinary pigment as a salt of a heavy metal (Pb, Cu, Hg, Sn, or Ag) has been opposed strenuously by Liebermann (24) on the ground of obtaining highly contaminated products. As evidenced by the Krüger-Wulff and Krüger-Schmidt (25) methods for the determination of purines, there can be no doubt that purine and other contaminants must have been present in Dombrowski's copper precipitate (12).

Adsorption of the urinary pigment by means of charcoal (17, 18) and dialysis (19) have no theoretical drawbacks so far as the integrity of the pigment is concerned. The previous concentration of the urine is necessary, however, in both of these methods. These physical procedures are not useful in the early process of separating the pigment from the bulk of urine.

The saturation of the urine with $(NH_4)_2SO_4$, followed by extraction with absolute alcohol—Garrod's technique (13)—is the best method thus far introduced for the preparation of the urinary pigment. However, the following objections to this method must be considered. It is impractical to saturate very large volumes of urine with $(NH_4)_2SO_4$. Ethyl alcohol is a good solvent for many substances in the urine other than urochrome, notably urea. The process of purification involves lengthy washing with water, so that a considerable loss of pigment is unavoidable.

## EXPERIMENTAL.

The development of an improved technique for the isolation of the normal urinary pigment appeared to be in the direction of finding a suitable solvent with which to extract urochrome *directly* from urine.

Table I is practically self-explanatory. It indicates the conditions under which various solvents were found to extract pigment from urine. With the exception of *n*-butyl alcohol (where a slight extraction of pigment occurred), none of the solvents tried extracted any color from untreated urine. The effect of $(NH_4)_2SO_4$ was not due to its acidity, for ethyl alcohol extracted the pigment from urine, saturated with $(NH_4)_2SO_4$ and rendered strongly alkaline by the addition of NaOH (26). No correlation between the solubility of the solvents in water and the extraction of pigment from acidified urine was demonstrated, although the best extractants of urochrome under these conditions—normal and isobutyl alcohols and isobutyl aldehyde—have very similar solubilities (27).

TABLE I.

*Extraction of Urinary Pigment with Various Solvents.*

| Solvent. | Solubility in $H_2O$.* | Extraction of pigment. | |
|---|---|---|---|
| | | After saturation with $(NH_4)_2SO_4$. | After acidification of urine. |
| Ethyl alcohol | Soluble in all proportions. | ++++ | 0 |
| Ether | $6.48^{20°}$ | 0 | 0 |
| Chloroform | $0.617^{22°}$ | 0 | 0 |
| Ethylene chloride | $0.861^{20°}$ | + | 0 |
| Methylethylketone | $22.6^{20°}$ | + | 0 |
| Ethyl acetate | $8.27^{20°}$ | +++ | 0 |
| “ butyrate | $0.5^{22°}$ | ++ | 0 |
| Butyl acetate | $0.5^{22°}$? | ++ | 0 |
| “ alcohol (normal) | $7.81^{20°}$ | ++++ | ++++ |
| “ “ (secondary) | $22^{20°}$ | +++ | ++ |
| Isobutyl alcohol | $9.5^{18°}$ | ++++ | ++++ |
| Butyl alcohol (tertiary) | Soluble in all proportions. | + | 0 |
| Isobutyraldehyde | $10^{20°}$ | +++ | +++ |
| Amyl acetate | $0.18^{20°}$ | + | 0 |
| “ alcohol (normal) | $2.7^{22°}$ | ++ | + |
| Benzene | $0.072^{22°}$ | 0 | 0 |
| Phenol | $8.4^{20°}$ | + | 0 |

* Figures are based on the data in volumes I of Landolt-Börnstein and International Critical Tables (see also reference 27).

0 indicates no extraction of pigment; +, slight extraction; ++++, maximum extraction.

At 20° butyl alcohol contains approximately 20 per cent of water and water contains about 8 per cent of butyl alcohol when the alcoholic and aqueous phases are in equilibrium with each other (28). Dakin (29) considered the solubility of water in butyl alcohol essential in the extraction of the monoamino acids. After preliminary concentration of a butyl alcohol extract of urine, it was dehydrated with successive liberal quantities of anhydrous sodium sulfate, barium perchlorate, and finally by the use of calcium carbide. The water-free extract was highly colored and presumably little pigment was lost during the successive stages of dehydration. The urinary pigment was soluble, therefore, in water-free butyl alcohol.

A series of experiments, which invariably yielded the same data, indicated that above pH 4.5 practically no pigment was extracted from fresh urine by *n*-butyl alcohol. At this acidity extraction definitely began and approached a maximum at pH 3.9. Therefore, for optimum extraction of pigment urine should be acidified to pH 4.3 to 3.9. Old, putrid urine was found refractile to extraction (of pigment) with this solvent, even after proper acidification.

With increase in alkalinity, at pH 6.8 the pigment just began to pass into water from a water-washed butyl alcohol extract. At pH 7.2 about one-third of the coloring matter had shifted to the aqueous layer. At pH 7.5 there was approximately an equal distribution of pigment between water and butyl alcohol, while at pH 8.0 most of the pigment had left the butyl alcohol.

The pigment from another sample of the above butyl alcohol extract was transferred to water at approximately pH 9.0. With decreasing alkalinity, at pH 6.8 there was a sudden shift of the greater part of the pigment from the aqueous to the alcoholic phase. At pH 6.0 most of the pigment had passed into the butyl alcohol, while at pH 5.8 only a trace of coloring matter remained in the water. At pH 5.5 to 5.4 the aqueous layer appeared completely decolorized. Thus, in a separatory funnel containing water and butyl alcohol the pigment could be made to pass at will from one solvent to the other by changing the hydrogen ion concentration. It is to be noted that under the above conditions maximum extraction of pigment by butyl alcohol was at a higher pH than in the original extraction of pigment from whole urine.

Butyl alcohol removed about 40 per cent of the coloring matter from urine in a single extraction and, when the acid reaction was maintained, about 80 per cent in four successive extractions, provided that the urine was concentrated to a small volume before the last extraction. The pigments in the four extracts behaved identically in reference to solubility in water and butyl alcohol with changes in pH and had similar light absorption properties (spectroscopically). During the course of this work about 1200 liters of urine have been submitted to the process of extraction (a single, rapid shaking with *n*-butyl alcohol). No attempt was made to recover the solvent which dissolved in the urine during the extraction period. The extract was concentrated *in vacuo* and the butyl alcohol recovered in the distillate was used again. The total loss of butyl alcohol has been less than 60 liters, or, roughly, about 50 cc. per liter of urine.

A very important advantage in the present method of extracting the normal urinary pigment is the fact that the butyl alcohol extract may be thoroughly washed with water. The unconcentrated extract was in some instances washed three times with double its volume of water in each washing with only a small loss of pigment. The amount of pigment lost in four successive washings with an equal volume of water was entirely negligible. Acid was removed by the washing. Salts, such as phosphates, and any extracted urea should be effectively removed by this washing process.

The essential details of the new method of preparing the urinary pigment were as follows: Urine was collected under toluene in six 18 liter bottles, through large funnels. As soon as possible after collection, the toluene was carefully siphoned off and the urine acidified by the addition of 150 cc. of glacial acetic acid to each 18 liter bottle of urine. To each bottle was now added about 1.5 liters of *n*-butyl alcohol and the direct extraction of pigment was accomplished by vigorous shaking for 5 to 10 minutes (insuring the complete admixture of solvent and urine). During the shaking a temporary emulsion of butyl alcohol extract and urine was formed. The bottles were permitted to stand for a period of time (up to 2 hours) to allow for the separation at the surface of the clear extract and emulsion. The whole upper layer (*including the emulsion*) was then siphoned off and filtered into tall cylinders through large fluted filters. The filtration process broke the emulsion effectively and two clear layers were obtained: a lower layer of pale, extracted urine and upper layer of extract, which, depending upon concentration, varied in color from a very deep orange to an intense Burgundy red. The alcoholic extract was separated by means of large separatory funnels. About 4 liters of extract were obtained from each 100 liter batch of urine.

The extract was washed four times by vigorous shaking in the separatory funnel with equal volumes of water. The washings were discarded and the extract concentrated *in vacuo* (temperature not exceeding 50°) to about one-third its original volume. The concentrated extract was put in the refrigerator for several days and then filtered. Very little material separated from the extract in the cold. For example, no crystals of uric acid could be found in the slight sediment. This was in marked contrast to the behavior on ice of extracts obtained by "continuous" extraction *in vacuo*, a method employed early in this work.

The extract was now washed by shaking with equal volumes of chloroform, benzol, and ether (to remove possible extraneous pigments, etc. (1) ), and was further concentrated to a small volume *in vacuo* (temperature 37°). At this stage the original 4 liters of extract had been reduced to a volume of about 600 cc. and pigment had begun to separate from the extract. The separation of pigment was most likely due to exceeding its saturation in the butyl alcohol. The extract was permitted to stand for several days and the separated pigment was collected upon a Buchner filter. The pigment was washed on the filter with chloroform, benzol, amyl acetate, and amyl alcohol (1). It was obtained in the form of a dry, brown powder by washing with anhydrous absolute alcohol and anhydrous ether, and was further dried in a vacuum desiccator over sulfuric acid.

After this first separation of pigment, the butyl alcohol concentrate has been worked up in one of several ways. In each method, dry pigment products were obtained which had properties (solubility, etc.) similar to the pigment discussed above.

(*a*) The extract was still further concentrated *in vacuo* resulting in a further separation of pigment, which was dried by washing with anhydrous absolute alcohol and anhydrous ether.

(*b*) The pigment from the butyl alcohol concentrate was transferred to water by alkalization (see p. 436). In some instances the aqueous solution was acidified, the pigment reextracted by means of butyl alcohol, and then once again transferred to water by increasing the pH. The alkaline aqueous solution was partially neutralized and concentrated to a small volume *in vacuo.* About 3 volumes of anhydrous ether were then added in the cold thereby precipitating most of the pigment. This precipitate was quickly collected upon a Buchner filter and dried in the manner indicated above. The precipitated pigment was not left in contact with ether too long, as changes in its solubility and drying properties occurred when this was done.

(*c*) A fairly efficient precipitation of pigment from the butyl alcohol concentrate has been obtained by the direct addition to it in the cold of anhydrous absolute alcohol or anhydrous ether. The pigment was collected and dried in the usual manner.

Some possible disadvantages of the butyl alcohol extraction method may be: (*a*) Upon standing a number of days there was an apparent increase in the depth of color of the butyl alcohol extract. Definite changes in the properties of the pigment took place when the butyl alcohol extract was permitted to stand several months or longer. The pigment, for example, became soluble in chloroform and resisted transfer into water except in the presence of strong alkali. These changes may possibly have been due to esterification. The extract was, therefore, "worked up" quickly.

(*b*) Besides the normal pigment of the urine other pigments may be extracted by butyl alcohol. The only report in the literature upon the use of butyl alcohol for the extraction of pigment is the interesting paper of Rosenheim (30). This investigator has found that butyl alcohol will extract all the anthocyanins from their solutions in dilute acids. The pigment of beetroot was not extracted and is probably not a true anthocyanin.

A preliminary study has been made of the following pigments.

*Acid Hematin.*—This pigment was extracted from 0.1 N HCl by butyl alcohol. When the acid hematin solution was rendered alkaline, pigment was not extracted by the organic solvent. Upon reacidification the pigment was once again extractable.

*Bilirubin.*—Butyl alcohol did not extract bilirubin from 0.1 N $NH_4OH$. Upon acidification, however, the pigment was quantitatively extracted. Upon standing about 24 hours the color of the alcoholic solution changed from yellow to green (biliverdin?) and upon longer standing from green to blue. The biliary pigment could not be transferred to water from the butyl alcohol by increasing the pH. Thus, urochrome and bilirubin, if present together, may be effectively separated by the alkalization of the butyl alcohol solution of the two pigments. Washing the urinary pigment with chloroform assures the removal of traces of bile pigment.

*Carotin.*—The writer has already reported the finding that butyl alcohol will extract carotin directly from untreated serum (31). Carotin, like bilirubin, cannot be transferred to water. It is soluble in the fat solvents and presents no problem in the purification of the urinary pigment.

## DISCUSSION.

The most imporant factor in the extraction of the normal urinary pigment by means of butyl alcohol is the hydrogen ion concentration. The use of mineral acids and acidities below pH 3.0 must be avoided, since the pigment is unstable under these conditions. Acidification is readily obtained with acetic acid, since maximum extraction is at pH 3.9.

The ability to shift the urinary pigment from butyl alcohol to water and back with change in pH may serve as an important means of purification. This characteristic property may also be used in preliminary identification of related pigments (such as the pigment in the aqueous extract of muscle (31)).

## SUMMARY.

The older methods of preparing the normal pigment of the urine have been found inadequate.

A new method of extracting the pigment by means of *n*-butyl alcohol has been described. Butyl alcohol is especially useful in this connection because it is a very poor solvent for most of the inorganic constituents of the urine and, at room temperature, also for uric acid and urea. In the present method of purifying the extract only the gentlest procedures have been employed.

The importance of pH in the differential solubility of the urinary pigment in butyl alcohol and water has been discussed.

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# THE NORMAL PIGMENT OF THE URINE.

## IV. PRELIMINARY STUDY OF THE PROPERTIES OF THE PIGMENT OBTAINED BY THE NEW METHOD OF BUTYL ALCOHOL EXTRACTION.*

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In the preceding paper (1) a new method of preparing the normal pigment from urine has been described. It consisted essentially of extracting the pigment from acidified urine (pH about 4.0) with *n*-butyl alcohol, washing freely at different stages in the procedure with water, chloroform, benzol, amyl acetate, and amyl alcohol and finally obtaining the pigment product in a dry form by washing with anhydrous absolute alcohol and anhydrous ether.

The purpose of this communication is to describe briefly a number of preliminary studies which indicate that the normal urinary pigment has not been altered significantly during the course of preparation and that it has been prepared in probably a higher state of purity than hitherto. Some further evidence also has been adduced to show that the pigment is a chemical entity and not a mixture of substances.

### EXPERIMENTAL.

With the new method, the pigment was obtained in the form of a dry, brown powder, which no longer had the odor of urine. Microscopically, the freshly prepared precipitate of the urinary pigment in a drop of the mother liquor was usually refractile in character, and in a number of preparations, the pigment particles looked like very small needles and could be called semicrystalline.

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* A preliminary report of part of this work has appeared in the Proceedings of the American Society of Biological Chemists (Drabkin, D. L., *J. Biol. Chem.*, **74**, p. xv (1927)).

Extraneous pigments, such as carotin, bile pigments, urobilin, and hematoporphyrin, could not be demonstrated spectroscopically in solutions of the purified pigment. The absence of uric acid was indicated by negative murexide tests upon the dried preparations. The method of preparation—extraction with butyl alcohol at room temperature and copious washing with water—precluded the presence of any except possibly minute traces of urea. The ammonia production, if any, from the action of urease upon 0.2 gm. of purified pigment, dissolved in water and digested at pH 6.8 (adjusted by buffer), could not be detected.

The physical properties of the pigment were: (*a*) The solubilities of the freshly prepared pigment were found to be practically identical with those of the pigment prepared by Garrod (2). It was very soluble in cold and hot water, reproducing the original color of the urine in these solutions. (The tint of aqueous solutions varied from golden yellow to reddish brown, depending on the concentration.) The pigment preparation was also very soluble in dilute ethyl alcohol, though much less readily soluble in commercial "absolute" alcohol. It was relatively insoluble in anhydrous alcohol and insoluble in anhydrous ether, chloroform, acetone, and benzene. The pigment was somewhat soluble in alcohol-ether and alcohol-chloroform mixtures. On standing 1 year over sulfuric acid in a vacuum desiccator, the pigment became relatively insoluble in cold water, although it was still fairly soluble in hot. This change in solubility was not accompanied by any change in physical appearance.

(*b*) Aqueous and alcoholic (ethyl and butyl alcohol) solutions of the pigment exhibited the absorption spectrum characteristics of a neutral filter, showing a uniform absorption in the violet and ultra-violet regions. In solutions in which the intensity of color was about two times that of normal urine, absorption began at 4560Å. and was strong at 4250Å. (violet region of spectrum). In more concentrated solutions there was slight absorption of light in the blue-green (beginning at 5430Å.), with definite absorption at 5000Å. Except for a displacement of the absorption about 50Å. towards the violet in the case of butyl alcohol solutions there was no difference between the spectral characteristics of the pigment in the above solvent and (in approximately similar concentration) in ethyl alcohol. During various stages of purification no change in spectrum was observed, definite absorption in dilute solutions still beginning at about 4250Å. Had urobilin been present in these solutions it would have been recognized by its absorption band (approximately 5000 to 4900Å.). Findings in close agreement with the above were also obtained photographically, with a quartz spectrograph.

Moderately concentrated solutions of the pigment exhibited the property of fluorescence between 3680 and 3122Å. This phenomenon was brought out by a Wratten 18 filter and a mercury arc lamp. Although the characteristic 2-banded spectrum of urobilin did not appear, an aqueous solution of urochrome, illuminated by a Welsbach lamp, fluoresced strongly after treatment with $NH_4OH$ and $ZnCl_2$ (followed by filtration). A strongly fluorescent solution (absorbing light definitely from 4120Å. on) was also obtained after heating an aqueous solution with glacial acetic and iodic acids. The urinary pigment, however, was probably destroyed by this treatment.

(*c*) The pigment in aqueous solution did not dialyze through parchment, either into water or, when acidified, into butyl alcohol.

(*d*) A thoroughly washed, concentrated butyl alcohol solution of the pigment was subjected to "chromatographic analysis" by Tswett's method (3). In this method, when a solution of several pigments is slowly washed through a column of adsorbent such as anhydrous calcium carbonate, the pigments, due to their relative affinities for the adsorbent, are approximately separated in layers. This type of analysis indicated that the pigment preparation is not a mixture of substances. In control tests, bilirubin and carotin,[1] when mixed with the urinary pigment, were readily separated by this technique.

The chemical properties of the pigment were:

(*a*) The biuret reaction upon nearly colorless solutions of the purified pigment was negative. A very faint, unsatisfactory Adamkiewicz reaction was obtained. The Millon test, on the other hand, was consistently fairly strongly positive.

(*b*) The pigment from a washed butyl alcohol extract was transferred to water at approximately pH 9. From this solution the following metals were found to precipitate the pigment: $Ag(NO_3^-$

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[1] Very kindly supplied by Professor Frank P. Underhill of Yale.

and $SO_4^{=}$), $Ba(Cl^{-})$, $Cu(SO_4^{=})$, $Fe(SO_4^{=}$ and $Cl^{-})$, $Hg(NO_3^{-}$ and $Cl^{-})$, $Pb(CH_3COO^{-})$, and $Zn(Cl^{-})$. The Cu, Fe, Hg, and Zn "salts" of urochrome were soluble in HCl, while the Ag precipitate was not. As originally found by Dombrowski (4) in the case of his copper-urochrome preparation, the Ag and Cu "salts" were soluble in $NH_4OH$, the former producing a Burgundy red solution, the latter a blue-green. In two instances, with the compounds of Cu and Fe, refractile crystalline-like products were seen under the microscope, after careful evaporation of their ammoniacal and acid solutions.

(*c*) The statement in the preliminary report that the urinary pigment, prepared by the new method, was not precipitated by alkaloidal reagents must be modified to include a more extended experience. The above statement was based upon observations made upon an aqueous pigment solution, freshly transferred from butyl alcohol at pH 6.8. Under these circumstances, the urinary pigment was not precipitated by the addition of phosphotungstic, trichloroacetic, chromic, or picric acids.

The writer has recently found that the reaction of the solution must be very carefully adjusted, because urochrome is precipitated by phosphotungstic and silicotungstic acids only in a narrow range of pH. No precipitation took place when the solution was too alkaline or too acid. The optimum pH was found to be 3.8 and was insured by the use of a buffer solution. To a buffer-adjusted solution containing a weighed quantity of purified pigment 10 per cent silicotungstic acid was gradually added until the appearance of turbidity. Upon standing overnight in the refrigerator, the solution was appreciably decolorized with the formation of a dark brown precipitate, which was beautifully crystalline under the microscope. The crystals were quadrangular, flat plates, or prisms, their color presumably varying with thickness from a pale yellow-pink to a red-brown. Thus far, however, all preparations have contained some crystals which appeared to be practically colorless.

(*d*) Dombrowski (4) was the first to show that urochrome possesses reducing properties. That the pigment prepared by the new method is a mild reducing agent was suggested by the appearance of a blue color upon the addition of a solution of the pigment acidified with glacial acetic acid to a dilute solution of ferric chloride plus a dilute solution of potassium ferricyanide. Iodic acid, on the other hand, was not reduced, nor were other tests for reduction positive.

(*e*) An interesting, new observation was the complete decolorization of the pigment solution by heating with zinc dust and HCl, although whole urine could not be rendered completely colorless by this means. The aqueous pigment solution was also appreciably but not totally decolorized by treatment with sodium hydrosulfite. Bubbling a stream of hydrogen through the pigment solution produced no changes, nor was hydrogen rendered effective by the presence of platinized asbestos. Thus, decolorization was produced only with very powerful reductants.

Upon standing exposed to the air, the decolorized solution very gradually took on color. In the presence of hydrogen peroxide the restoration of color was very rapid. It should be noted, however, that, while the intensity of the restored color was equal to the original, the tint was appreciably changed—the new color being pinkish. The decolorization and restoration of pigment could be repeated a number of times upon the same solution, although there was evidence of loss of pigment during the process.

(*f*) Contrary to Dombrowski's findings (4), the non-specific sodium nitroprusside reaction (for the SH group) was consistently negative, both before and after the reduction of the pigment with zinc and hydrochloric acid. Although a faint, pinkish color was sometimes produced after the addition of the nitroferricyanide reagent, fading of color was not observed after the addition of glacial acetic acid. In only one instance out of a great many was a good test for soluble sulfides obtained (by precipitation as PbS), after the incineration of the pigment with metallic sodium.

Due to the unreliable nature of the above tests, the S was determined quantitatively by Osborne's (5) peroxide fusion method. In a single determination, 0.5 gm. of urinary pigment was found to contain 0.0032 gm. of sulfur (or 0.64 per cent). An equal quantity of high quality casein, run as a control, contained 0.0043 gm. of sulfur (or 0.86 per cent). No correction was made for the sulfur impurities in the reagents, the correction being negligible since only comparative values were sought.

The pigments of dog and rat urine could be extracted by *n*-butyl alcohol and maximum extraction took place at practically the same pH (3.9) as in the case of human urine (1). With change in hydrogen ion concentration, these pigments also could be transferred from butyl alcohol to water and back.

It was of interest to evaluate provisionally the empirical standard which the writer had used in his earlier studies (6) in terms of mg. of purified pigment. A dilute alkaline solution of a weighed quantity of pigment was compared colorimetrically against the alizarin-aniline orange standard. 1 unit of pigment was found to be equivalent to 3.82 mg. of pigment. An average pigment output for an adult male was calculated to be 73 mg. (from 19.0 units) per 24 hours. On the same basis the output per square meter of surface area was calculated to be 42 mg. (from 11.0 units). Dombrowski's (4) figures for the daily excretion of urochrome are 6 to 10 times the above.

## SUMMARY.

A preliminary study of the physical and chemical properties of the normal urinary pigment, prepared by butyl alcohol extraction, has indicated that the coloring matter of urine has been obtained relatively unchanged and free from most adventitious contaminants.

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# APPLICATIONS OF STATISTICAL METHOD TO THE DATA OF VITAMIN FEEDING EXPERIMENTS.

## I. THE PER CENT EFFECT OF MEASURED VARIABLES.*

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### INTRODUCTION.

In biological researches, especially those involving the use of experimental animals, the results obtained are often of such irregularity that the interpretation of the data is a perplexing part of the investigation. This is, in large measure, due to that element termed individual variation which is an admission of the fact that we are as yet unable to control many of the factors affecting our results. The biologist does not have at his command the precision of technique employed by the chemist or the physicist. It is therefore advisable in biological work to use statistical method as an aid in the interpretation of data in order to eliminate, in so far as possible, unwarranted conclusions due to chance variations. Statistical method can also be used as a tool in determining the general trend of reactions now on record with a view to making predictions as to future reactions. Such predictions must, of course, be verified by experimental data.

The methods of vitamin assay are based upon the theory that when all food essentials except one vitamin are present in the diet in adequate amounts, the gain in weight is due to the vitamin added as a supplement. Even when performed under carefully standardized technique the gains in weight made by the experimental animals show considerable variability. This variability

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is not peculiar to our laboratory but is a problem of general concern. It was thought advisable to make a statistical study of the data now on record in this laboratory with a view to determining, if possible, the causes of the variability in our animals and to enable us to know where to begin in attempting to render our technique more accurate and our results less variable. In this paper are reported the means and correlations of different variables, together with the per cent effect of certain measured variables on the gain in weight.

## EXPERIMENTAL.

The vitamin tests performed in this laboratory have had to do largely with the effect of cultural conditions and degree of maturity upon the vitamin content of vegetables. For the purpose of this study, it has been assumed that the animals received the same quantity of vitamin, which is not strictly true, as the animals have been fed different kinds and different amounts of vegetables as the source of the vitamin under investigation. It may be stated here that in all our vitamin work an attempt has been made to approach the maintenance level for vitamin B tests and the 3 gm. gain per week for vitamin A tests, so that the data are not as heterogeneous as might be supposed at first thought.

The animals used in these tests were albino rats 28 days of age. It has been shown by several investigators that the diet of the mother rat during pregnancy and lactation is a determining factor in the growth response of the young rats used for vitamin tests. The rats whose records were used in this study were not all reared on the same basal diet. Approximately 75 per cent of the animals were reared in this laboratory, where it is standard practice to reduce all litters to eight rats. The mothers of these animals received a modified form of Steenbock's basal ration which has the following composition: yellow corn-meal 64, linseed oil meal 16, yeast 2, wheat germ 10, crude casein 5, ground alfalfa 2, NaCl 0.5, and $CaCO_3$ 0.5. 10 cc. of fresh whole milk were fed daily. The other 25 per cent of the animals were supplied by our Chemistry Department. The mothers of these animals were fed a diet of the following composition: oat groats 35, yellow corn 38, alfalfa 5, wheat 9, tankage 6, linseed oil meal 5, and dried buttermilk 3.

The technique used was essentially that of Ferry (1) each rat being kept in an individual all metal cage with a false bottom. The basal diet and distilled water were fed *ad libitum* and the daily allotment of the vitamin-containing food was fed separately. Daily food consumption records were kept and the animals weighed once each week or more often. The data obtained from these experiments were tabulated for this statistical study under the following headings: animal number, strain, sex, initial weight, final weight, total experimental gain, days on experiment, vitamin studied, weight of the vitamin-containing food, kind of vitamin-containing food, condition of vitamin-containing food, total basal diet, kind of basal diet, and average daily consumption of the basal diet. In the case of vitamin A experiments, the animals were

TABLE I.

*Composition of the Four Basal Diets.*

| | Sherman and Munsell vitamin A-free diet. | Osborne and Mendel vitamin A-free diet. |
|---|---|---|
| | *per cent* | *per cent* |
| Vitamin A-free casein | 20 | 18 |
| Starch | 70 | 51 |
| Yeast | 5 | 5 |
| Salts (6) | 4 | 4 |
| Crisco | | 22 |
| NaCl | 1 | |
| Calories per gm. diet | 3.6 | 4.7 |

| | Sherman and Spohn vitamin B-free diet. | Osborne and Mendel vitamin B-free diet. |
|---|---|---|
| | *per cent* | *per cent* |
| Vitamin B-free casein | 18 | 18 |
| Starch | 68 | 50 |
| Butter fat | 8 | 8 |
| Salts (6) | 4 | 4 |
| Lard | | 18 |
| Cod liver oil | 2 | 2 |
| Calories per gm. diet | 4.3 | 5.2 |

depleted of their body store of vitamin A but only the data of the experimental period were used.

During the course of the experimental work, two basal diets have been used for the vitamin A tests and also for the vitamin B[1] tests. In the case of the vitamin B experiments, the diets were those of Sherman and Spohn (2), and Osborne and Mendel (3). For the vitamin A experiments the diets were those of Sherman and Munsell (4) and Osborne and Mendel (5). The composition of each of these four rations with their approximate caloric value per gm. of the basal diet is given in Table I. The chief difference between the two vitamin A-free diets and between the two vitamin B-free diets is in the amount of fat they contain.

### *Comparisons of Means and Coefficients of Variability.*

The data of both the vitamin A and B experiments were separated into groups according to the basal diet fed, thus making four groups. Means were calculated in order to detect any striking differences among any of the four groups of animals. The means, standard deviations, and coefficients of variability are shown in Tables II and III.

It will be seen in Table II that the mean initial weights with their standard deviations and coefficients of variability are practically the same for both diets. The average number of days required for depletion is less on the Osborne and Mendel diet containing fat than on the Sherman and Munsell fat-free diet, the ratio of the mean difference to its standard deviation being 5.4. In vitamin A experiments the shorter depletion period is advantageous from the standpoint of economy of time, laboratory space, and expenditure for food materials. It also places the animals on experiment at a lower age level and prevents the experimental period from extending beyond the logarithmic phase of the growth curve. By examining Table II further, it will be seen that during the experimental period the gain in weight on the Sherman and Munsell fat-free diet is greater than on the Osborne and Mendel diet containing fat. In this case the ratio of the mean

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[1] Throughout this paper the term vitamin B refers to the associated antineuritic and growth-promoting vitamins as they occurred in the vegetables under examination.

TABLE II.

*Mean Initial and Final Weights, Standard Deviations, and Coefficients of Variability of Rats Fed Different Basal Diets Free of Vitamin A.*

| | Sherman and Munsell vitamin A-free diet. | Osborne and Mendel vitamin A-free diet. |
|---|---|---|
| No. of rats | 120 | 349 |
| Mean initial weight, *gm.* | 110 ±2.0 | 112 ±1.1 |
| Standard deviation of initial weight, *gm.* | 32 | 31 |
| Coefficient of variability of initial weight, *per cent.* | 29 | 28 |
| Mean No. of days on depletion. | 46 ±0.7 | 40 ±0.3 |
| Standard deviation of days on depletion | 11 | 8.9 |
| Mean final weight, *gm.* | 161 ±2.8 | 144 ±2.0 |
| Standard deviation of final weight, *gm.* | 46 | 55 |
| Coefficient of variability of final weight, *per cent.* | 28 | 38 |
| Mean gain, *gm.* | 51 ±2.5 | 32 ±1.9 |
| Standard deviation of gains in weight, *gm.* | 40 | 53 |
| Mean calories eaten during the experimental period. | 1902 | 1458 |
| Gm. gained per gm. food ingested | 0.097 | 0.104 |
| " " " calorie ingested. | 0.027 | 0.022 |

TABLE III.

*Mean Initial and Final Weights, Standard Deviations, and Coefficients of Variability of Rats Fed Different Basal Diets Free of Vitamin B.*

| | Sherman and Spohn vitamin B-free diet. | Osborne and Mendel vitamin B-free diet. |
|---|---|---|
| No. of rats | 130 | 330 |
| Mean initial weight, *gm.* | 51 ±0.5 | 43 ±0.2 |
| Standard deviation of initial weight, *gm.* | 9 | 6 |
| Coefficient of variability of initial weight, *per cent.* | 17 | 14 |
| Mean final weight, *gm.* | 81 ±2.4 | 59 ±0.8 |
| Standard deviation of final weight, *gm.* | 40 | 21 |
| Coefficient of variability of final weight, *per cent.* | 49 | 43 |
| Mean gain, *gm.* | 30 ±2.3 | 15 ±0.8 |
| Standard deviation of gains in weight, *gm.* | 39 | 22 |
| Mean calorie intake during experimental period | 1033 | 978 |
| Gm. gained per gm. food ingested | 0.127 | 0.080 |
| " " " calorie food ingested | 0.029 | 0.015 |

difference to its standard deviation is 4.3. The efficiency of the two diets calculated as gm. gained per gm. of diet consumed and the gm. gained per calorie of diet consumed can also be seen from the table. Whether the difference in gain is due to the difference in calorie intake or to some metabolic property of the fat itself is a question. In a paper dealing with the nutritive value of different fats, Takahashi (7) states that vitamin A plays an important rôle in the combustion of fat in the animal tissue. He suggests that the body demands vitamin A in proportion to the amount of fat consumed and vitamin B in proportion to the amount of carbohydrate consumed. These data would confirm this view in that upon the same quantity of vitamin A less gain in weight is made when the basal diet contains fat. Is vitamin A used in the metabolism of the fat so that the animals were actually receiving less vitamin A for growth? It will also be observed that whereas the initial weights and their standard deviations are practically the same on both diets, the standard deviation of the final weights of the animals fed the Osborne and Mendel basal diet is greater than that of the animals fed the Sherman and Munsell basal diet. The ratio of the mean difference to its standard deviation in this case is 4.7. What is the cause of this variability? Were these animals using their vitamin A for fat metabolism and consequently receiving less for protection against infections? Does this susceptibility to infection account for the greater variability in their reactions? If the animals making smaller gains are more variable in their reactions is a minimum gain of 3 gm. per week a desirable standard?

Table III is similar to Table II, except that the data deal with the vitamin B experiments.

The differences between the mean initial weights, the standard deviations, and coefficients of variability, of the vitamin B test animals, as shown in Table III, are possibly influenced by the fact that the 130 animals fed Sherman and Spohn vitamin B-free diet were kept on a depletion period of 2 weeks before the experiment proper was begun. This work was done before the paper of Sherman and MacArthur (8), showing the futility of this procedure, was published. The group of rats fed the vitamin B-free diet containing the higher percentage of fat gained less, and were somewhat less variable in their reactions than the other group. The ratio of the mean difference in gain to its standard deviation is 4.1 in this case. If these differences are biologically significant, their cause is not understood.

An interesting observation regarding the data presented in Tables II and III is the difference in the coefficients of variability of the initial and final weights. The only group which does not show an increase in the coefficient of variability during the experimental period is the vitamin A group fed the Sherman and Munsell basal diet. To what is this increase in variability during the experimental period due? Is it due to uncontrollable physiological factors only or is it possible to perfect our technique sufficiently so that the weights of our animals will not be more variable at the end than at the beginning of the experiment? Under present conditions, with the wide variations in gain, especially in the case of the vitamin A experimental animals, it is impossible to discriminate between small differences in gain unless a very large number of animals are used.

TABLE IV.

*Correlation Coefficients and Their Probable Errors.*

| Variables. | Sherman and Munsell vitamin A-free diet. | Osborne and Mendel vitamin A-free diet. |
|---|---|---|
| | Correlation coefficients based on 120 animals. | Correlation coefficients based on 349 animals. |
| Initial weight and total food intake | 0.31 ±0.06 | 0.13 ±0.03 |
| " " " " gain | −0.20 ±0.06 | −0.20 ±0.03 |
| Total food intake and total gain | 0.73 ±0.03 | 0.83 ±0.02 |
| | Sherman and Spohn vitamin B-free diet. | Osborne and Mendel vitamin B-free diet. |
| | Correlation coefficients based on 130 animals. | Correlation coefficients based on 330 animals. |
| Initial weight and total food intake | 0.06 ±0.06 | 0.08 ±0.04 |
| " " " " gain | 0.05 ±0.06 | −0.17 ±0.04 |
| Total food intake and total gain | 0.86 ±0.01 | 0.79 ±0.01 |

### *Further Comparisons on the Basis of Gross Correlations.*

Correlations were run between the variables: initial weight, final weight, days on experiment, total food intake, and total gain. The more striking of these correlations are those between initial weight and total food intake, initial weight and total gain, total food intake and total gain. These correlations with their probable errors are recorded in Table IV.

In any one of the four groups the biological significance of the correlations between initial weight and total food intake and between initial weight and total gain is questionable but the correlation between total food intake and total gain is undoubtedly significant (9).

### *Multiple Regression Coefficients and Regression Equations.*

For each of the four groups of data a multiple correlation coefficient ($R$) and a regression equation were calculated according to the method of Wallaee and Snedecor (10). The independent variables were initial weight ($A$), days on the experiment ($C$), and total basal diet ($D$), and the dependent variable was total gain ($X$). The coefficient and equation for the data of the vitamin A tests wherein the animals received the Sherman and Munsell vitamin A-free diet are as follows:

*Vitamin A.*—Sherman and Munsell vitamin A-free diet.

$$R = 0.86$$

$$\bar{X} = 23 - 0.61\,A - 0.32\,C + 0.20\,D$$

This four variable regression equation[2] is interpreted in the following manner. The gain $\bar{X}$ increases: (1) 0.61 gm. per gm. decrease in initial weight; (2) 0.32 gm. per day decrease in the experimental period; (3) 0.20 gm. per gm. increase in the total basal diet.

Similar multiple regression coefficients and regression equations, all of which are interpreted in the same manner, for the other three groups of animals are as follows:

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[2] A complete discussion of linear regression both simple and multiple can be found in any elementary text on statistics or in the *Iowa State College Official Publication* (10).

*Vitamin A.*—Osborne and Mendel vitamin A-free diet.

$$R = 0.91$$

$$\bar{X} = 43 - 0.58\,A - 1.96\,C + 0.43\,D$$

*Vitamin B.*—Sherman and Spohn vitamin B-free diet.

$$R = 0.89$$

$$\bar{X} = -\,1.9 - 0.20\,A - 1.0\,C + 0.37\,D$$

*Vitamin B.*—Osborne and Mendel vitamin B-free diet.

$$R = 0.90$$

$$\bar{X} = 15.0 - 0.88\,A - 0.50\,C + 0.34\,D$$

TABLE V.

*Score Cards Showing the Per Cent of the Measured Effect of Initial Weight, Days on Experiment, and Total Basal Diet on Total Gain.*

| | Initial weight. | Days on experiment. | Total basal diet. | Sum. |
|---|---|---|---|---|
| Vitamin A. Sherman and Munsell vitamin A-free diet. | | | | |
| Partial regression coefficients | 0.48 | 0.10 | 0.95 | 1.53 |
| Scores or rate per cent | 31.4 | 6.5 | 62.1 | 100.0 |
| Vitamin A. Osborne and Mendel vitamin A-free diet. | | | | |
| Partial regression coefficients | 0.35 | 0.73 | 1.57 | 2.65 |
| Scores or rate per cent | 13.2 | 27.5 | 59.3 | 100.0 |
| Vitamin B. Sherman and Spohn vitamin B-free diet. | | | | |
| Partial regression coefficients | 0.04 | 0.37 | 1.15 | 1.56 |
| Scores or rate per cent | 2.6 | 23.7 | 73.7 | 100.0 |
| Vitamin B. Osborne and Mendel vitamin B-free diet. | | | | |
| Partial regression coefficients | 0.24 | 0.18 | 0.93 | 1.35 |
| Scores or rate per cent | 17.8 | 13.3 | 68.9 | 100.0 |

If we knew and could measure all of the factors affecting the gain in weight of an experimental animal and if all the relationships were linear, each multiple correlation coefficient would be 1.00. The difference between 1.00 and the square of the multiple correlation coefficient of any one group represents roughly the extent to which other uncontrolled and unmeasured factors, and curvilinearity affect the gain in weight. These multiple correlation coefficients are relatively high and represent our success in estimating the gain in weight from the three independent variables. In other words, they represent the extent to which the variables, initial weight, days on experiment, and total intake of basal diet influence the gain in weight. Score cards representing the per cent of the measured effect of each of the three independent variables on the dependent variable have been made according to the method of Wallace and Snedecor (10) and are given in Table V.

It can be seen from Table V that the amount of food ingested is by far the most important of these factors. In all four groups the per cent effect of the amount of basal diet eaten is consistently greater than that of the initial weight or days on the experiment.

Sherman (11) has suggested that the initial weights of vitamin A test animals be controlled by controlling the diet of the mother and by establishing certain arbitrary limits of weight for the experimental animals in any one laboratory. Unless the animal dies before the expiration of the experimental period the time limit proposed is 56 days. Since these two factors, initial weight and experimental period, have much less influence on the gain in weight than the amount of basal diet eaten would it not be well to consider some regulation of this factor? Surely the question is worthy of further study.

## SUMMARY.

An application of statistical method to the data of 929 vitamin A and B feeding experiments has been made with a view to determining the factors influencing the variability of the weight gains of the test animals. It has been found that the quantity of the basal diet consumed is the measured factor having the greatest influence upon the growth response. The data suggest that this might be a valuable point of attack in attempting to develop a technique, which will result in more uniform results.

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# APPLICATIONS OF STATISTICAL METHOD TO THE DATA OF VITAMIN FEEDING EXPERIMENTS.

## II. HOW MANY ANIMALS PER EXPERIMENTAL LOT.*

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(Received for publication, July 17, 1929.)

### INTRODUCTION.

In making a quantitative determination of the amount of vitamin in a food the usual method employed is to feed a basal diet free of one vitamin *ad libitum* and supplement this diet with a daily allotment of the food under examination. Varying quantities of the supplementary food are fed to groups of the standard test animals until that quantity is found which will produce a standard gain in weight in a given length of time. From these data are calculated the units of vitamin in the food. Again, if it is desired to compare the vitamin potency of two foods, two grades of cod liver oil or two varieties of lettuce for instance, it is common practice to feed equal portions of the two supplements to comparable groups of standard test animals and compare the weight gains at the end of a specified time. In such an experiment, an attempt is usually made to approach a minimum rate of growth in order to keep the sensitivity of the method at a maximum (1–3).

When either of these two methods is used, a number of questions recur. How large a difference must be obtained between the weight gains of any two groups of animals before significance can be assigned to the results? What criterion shall be used to determine whether or not the difference in gain is due to the difference in treatment or due to chance variation? How many animals shall constitute an experimental lot? Obviously, if we could control

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all of the factors affecting the gain in weight of an animal then only one animal and a check would need to be used in any single experiment. Unfortunately, this is not possible and it becomes necessary to use relatively large groups of animals in order to describe the population accurately enough to discriminate between small differences in gain.

In order to describe more accurately the population of rats as found in this laboratory, without running an inconveniently large number of animals on any one dietary regimen, we have sorted out all of the data of comparable vitamin tests previously run, and, with the aid of multiple regression, we have estimated the standard deviations of the populations of the animals used for vitamin A and vitamin B tests. The results of this study are herein reported together with the method of calculation used so that a similar study could be made in another laboratory. It would be of interest to compare the variability of the animals of several laboratories with a view to discovering differences in treatment which might lead to improved technique.

## EXPERIMENTAL.

The project upon which we have been working for the past 3 years in this laboratory is entitled, "The Vitamin Content of Vegetables as Affected by Cultural Conditions and Degree of Maturity." During this time a large number of tests have been made using varying quantities of different vegetables as sources of vitamin A and vitamin B.[1] From the records of the animals so used have been separated a group of 349 animals used for vitamin A tests and 330 animals used for vitamin B tests. These animals were all reared in this laboratory, their parents being fed a slightly modified form of the stock ration recommended by Steenbock (4). It is common practice in this laboratory to limit all litters to eight rats. The young are started on the experiments at 28 days of age and weigh between 40 and 60 gm. Each rat is kept in an individual all metal cage with a false bottom. Daily food consumption records are kept and the rats are weighed once each week or more often. The 349 vitamin A test animals were fed Osborne and

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[1] Throughout this paper the term "vitamin B" refers to the complex—the associated antineuritic and growth-promoting factors.

Mendel's vitamin A- free basal diet (4) plus a weighed portion of a vegetable as the source of vitamin A. The mean gain in weight made by these animals during the 8 weeks feeding period was 32 ± 1.9 gm. The 330 animals used for vitamin B tests were fed Osborne and Mendel's vitamin B-free diet (4) plus a weighed portion of a vegetable as the source of vitamin B. The mean gain in weight of this group was 15 ± 0.8 gm. It was thought that these two groups of rats afforded an opportunity to study the variability of our animals in order to give us a view of the number of animals and differences in mean gains necessary to obtain results which are statistically significant.

In testing the significance of mean differences it is common statistical practice to hold to the criterion of a mean difference being three or more times as large as its standard deviation, *i.e.* $\frac{\mathrm{MD}}{\sigma_{\mathrm{MD}}}$ = 3 or more, wherein MD is the mean difference and $\sigma_{\mathrm{MD}}$ is the standard deviation of the mean difference calculated by the formula:[2]

$$\text{(a)} \qquad \sigma_{\mathrm{MD}} = \sqrt{\frac{\sigma_X^2}{n} + \frac{\sigma_Y^2}{n'}}$$

On application to a comparative vitamin test, MD becomes the difference in the mean gains of the two groups of rats, $\sigma_X$ the standard deviation[3] of the gains in weight of the individual animals in one group, $n$ the number in that group, $\sigma_Y$ the standard deviation of the gains in weight of the second group, and $n'$ the number of animals in the second group.

Whether or not a criterion of three times the standard deviation is too severe for biological work of this nature is a matter of con-

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[2] In the case of paired data the formula for calculating the standard deviation of the mean difference is:

$$\sigma MD \quad \sqrt{\frac{\sigma_X^2}{n} + \frac{\sigma_Y^2}{n'} - \left(\frac{\sigma_X}{\sqrt{n}}\right)\left(\frac{\sigma_Y}{\sqrt{n'}}\right) rX_Y}$$

but in this paper only unpaired data are discussed and formula (a) holds.

[3] The formula for the calculation of the standard deviation is $\sigma_X = \sqrt{\frac{\Sigma\,(X - M)^2}{n}}$ wherein $X$ = the gain in weight of an individual rat, $M$ the mean gain of the lot, and $n$ the number of animals in the lot.

troversy. Newcomb (5) states: "The criterion of three times the standard error is a severe one. With a difference of twice the standard error probable significance can be inferred." Fisher (6) states, "If the difference is many times greater than the standard error, it is certainly significant, and it is a convenient convention to take twice the standard error as the limit of significance." The adoption of any criterion must be tempered with judgment and an intimate knowledge of the details of the experiment. For the purpose of this study we have adopted the criterion $\frac{\text{MD}}{\sigma_{\text{MD}}} = 3$ as it was thought advisable to use a strict rather than a lenient standard.

For biological work in which statistical control is exercised, the standard error of estimate is probably a better representative of the standard deviation of the population than is the standard deviation of any one sample and should be used in calculating the standard deviation of the mean difference relative to determining the significance of differences.

The standard error of estimate may be calculated by the formula:

$$\text{(b)} \qquad \sigma_{X \cdot ACD} = \sqrt{\frac{\Sigma\,(X - M_X)^2}{n - m}\left(1 - R^2_{X \cdot ACD}\right)}$$

wherein $\sigma_{X \cdot ACD}$ is the standard error of estimate, $X$ the gain in weight of each animal, $M_X$ the mean of the $X$ values, $R$ the multiple correlation coefficient,[4] $n$ the number of observations, and $m$ the number of variables used in calculating $R$. The factors affecting the gain in weight which were measured during the course of the experiments discussed in this paper and which were used in calculating $R$ were: initial weight denoted by the symbol $A$; days on the experiment, $C$; and total diet, $D$. With the factors $A$, $C$, and $D$ as independent variables and total gain $X$ as the dependent variable, multiple correlation coefficients and regression equations were calculated for the animals used in the vitamin A tests and also for those used in the vitamin B tests. They are as follows:

*Vitamin A Test Animals.*—$R = 0.91$; $\overline{X} = 43.0 - 0.58A - 1.96C + 0.43D$.

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[4] The detailed method of calculating $R$, the multiple correlation coefficient, can be found in the bulletin "Correlation and Machine Calculation," by Wallace and Snedecor (Wallace, H. A., and Snedecor, G. W., *Iowa State College Official Pub.*, **23**, 36 (1925)).

*Vitamin B Test Animals*.—$R = 0.90$; $\overline{X} = 15.0 - 0.88A - 0.50C + 0.34D$.

The standard error of estimate (as calculated by formula (b)) is a better estimate of the standard deviation of the population from which we drew our sample, than a standard deviation calculated by the formula in foot-note 3, because we have eliminated by means of regression, the portion of the standard deviation due to the measured variables, in this case initial weight, days on the experiment, and food intake. In the standard error of estimate then, we have the portion of the standard deviation due to experimental differences and to unknown or unmeasured attributes, since by means of regression we have accounted for the variation due to controlled experimental differences.

In the case of the animals used for the vitamin A tests, $R = 0.9143$, $\Sigma(X - M_X)^2 = 977651.2720$, $n = 349$, and $m = 4$. Substituting these values in formula (b) above, we get:

$$\sigma_{X \cdot ACD} = 21.5622$$

which on the basis of the data at hand is our best estimate of the true standard deviation for the animals used for vitamin A tests in this laboratory. This statistic would not represent the variability of the animals used for vitamin A tests in any other laboratory colony as there are too many factors contributing to it which vary from laboratory to laboratory.

Substituting the standard error of estimate for the standard deviations in the formula for the standard deviation of a mean difference, formula (a), we obtain:

$$\text{(c)} \qquad \sigma_{\text{MD}} = \sqrt{\frac{\sigma^2_{X \cdot ACD}}{n} + \sigma^2_{X \cdot ACD}}$$

which, in case $n = n'$ becomes:

$$\text{(c}'\text{)} \qquad \sigma_{\text{MD}} = \sqrt{\frac{2\,\sigma^2_{X \cdot ACD}}{n}}$$

If we hold to the criterion of a mean difference that is 3 times its own standard deviation as worthy of significance, we can calculate the mean difference which must be obtained between two groups of rats in order to attach significance to the results. For example, suppose in our laboratory we have two groups of twenty rats each, which are used to test the vitamin A potency of two grades of cod liver oil. Substituting in formula (c′) we obtain:

$$\sigma_{MD} = \sqrt{\frac{(2)\ (21.5622)^2}{20}}$$

$$= 6.8181$$

Substituting this value in the equation MD = $3\sigma_{MD}$ we obtain MD = 20.4543.

TABLE I.

*Mean Differences in Weight Gains Necessary for Significance for Different Sized Groups of Animals Used for Vitamin A Tests.*

| No. of animals in group. | Standard error of mean difference. | Minimum mean difference for significance. |
|---|---|---|
| 6 | 12.4480 | 37.3440 |
| 8 | 10.7804 | 32.3412 |
| 10 | 9.6421 | 28.9263 |
| 12 | 8.8021 | 26.4063 |
| 14 | 8.1493 | 24.4479 |
| 16 | 7.6228 | 22.8684 |
| 18 | 7.1869 | 21.5607 |
| 20 | 6.8181 | 20.4543 |
| 22 | 6.5008 | 19.5024 |
| 24 | 6.2240 | 18.6720 |
| 40 | 4.8211 | 16.4633 |
| 104 | 2.9899 | 8.9697 |

In this table it is assumed that the means of one group closely approximate those of the other group.

It will be necessary, then, to obtain a mean difference in gain of 20 gm. in order to conclude that the difference in gain is due to the difference in the vitamin A content of the oils and not due to chance variation.

The above statements are true only in case the mean initial weight, mean food intake, and mean days on experiment of the two groups of twenty rats closely approximate each other.

The mean differences in weight gains which are necessary for significance for different sized groups of animals used for vitamin A tests in this laboratory have been calculated, with the above method and with the criterion, $\frac{\text{MD}}{\sigma_{\text{MD}}} = 3$. These mean differences are listed in Table I.

In the case of the animals used for the vitamin B tests $R = 0.8970$, $\Sigma(X - M_X)^2 = 157538.00$, $n = 330$, and $m = 4$. Substituting these values in formula (b) above we get $\sigma_{X \cdot ACD} = 9.7171$.

By use of the same method of calculation as for the vitamin A tests the mean differences in weight gains necessary for significance for different sized groups of animals used for vitamin B tests have been calculated. These mean differences are listed in Table II.

TABLE II.

*Mean Differences in Weight Gains Necessary for Significance for Different Sized Groups of Animals Used for Vitamin B Tests.*

| No. of animals in group. | Standard error of mean difference. | Minimum mean difference for significance. |
|---|---|---|
| 6 | 5.6101 | 16.8303 |
| 8 | 4.8586 | 14.5758 |
| 10 | 4.3455 | 13.0365 |
| 12 | 3.9670 | 11.9010 |
| 14 | 3.6726 | 11.0178 |
| 16 | 3.4355 | 10.3065 |
| 18 | 3.2390 | 9.7170 |
| 20 | 3.0728 | 9.2184 |
| 22 | 2.9298 | 8.7894 |
| 24 | 2.8050 | 8.4150 |
| 40 | 2.1728 | 6.5184 |
| 104 | 1.3475 | 4.0425 |

In this table it is assumed that the means of one group closely approximate those of the other group.

In case the means of the two samples are not nearly equal, the mean difference must be corrected before the above method becomes applicable. The correction is as follows:

Corrected $\text{MD}_X = \text{MD}_X - 0.58\,\text{MD}_A - 1.96\,\text{MD}_C + 0.43\,\text{MD}_D$ in which

$\text{MD}_X$ = The difference of the mean weight gains of the two samples or groups;

$\text{MD}_A$ = the difference of the mean initial weights of the two samples;

$\text{MD}_C$ = the difference of the mean number of days on experiment of the two samples;

$\text{MD}_D$ = the difference of the mean weight of the basal diet ingested of the two samples.

The coefficients in the correction equation are those in the regression equation for vitamin A test animals. The correction equation for the vitamin B test animals for this laboratory is:

$$\text{Corrected } MD_X = MD_X - 0.88\, MD_A - 0.50\, MD_C + 0.34\, MD_D$$

The following example illustrates the necessity for the correction equation and its use. In an article published last year (7) the senior author gave some results comparing the vitamin A content of ethylene-ripened, air-ripened, vine-ripened, and green tomatoes. The means of the four variables for each of the four classes of tomatoes were as follows:

| | Initial weight. | Days on experiment. | Total basal diet ingested. | Weight gained. |
|---|---|---|---|---|
| | *gm.* | | *gm.* | *gm.* |
| Ethylene-ripened | 124.10 | 56.00 | 598.05 | 58.40 |
| Air-ripened | 116.42 | 56.00 | 570.89 | 57.10 |
| Vine-ripened | 123.00 | 56.00 | 584.95 | 55.80 |
| Green | 122.81 | 56.00 | 583.52 | 38.50 |

The mean difference in weight gains between the ethylene-ripened and air-ripened tomatoes is 1.3 gm. which is not significant when tested by the usual test as was done in that paper. Upon applying the correction equation as suggested in this paper we have:

$$MD_X \text{ (corrected)} = 1.3 - (0.58)\,(7.68) - (1.96)\,(0) + (0.43)\,(27.16) = 8.5288$$

The corrected mean difference is considerably larger than the observed mean difference but is still not significant since, as is shown in Table I, a mean difference of 20.45 is necessary for significance for groups of twenty animals each.

The mean difference in weight gains between the ethylene-ripened and green tomatoes is 19.9 gm. which is bordering on significance by the usual test. The correction is

$$MD_X \text{ (corrected)} = 19.9 - (0.58)\,(1.29) - (1.96)\,(0) + (0.43)\,(14.53) = 21.10$$

which is in excess of the 20.45 gm. necessary for significance as shown in Table I.

The data in Tables I and II indicate that the animals used for vitamin B tests make more consistent weight gains than do the animals used for vitamin A tests. In vitamin B experiments the animals are placed upon the experimental diet at exactly 28 days of age and do not vary greatly in weight (4). Due to the depletion period used in vitamin A experiments the animals are started on the experimental diet at varying ages and are older at the beginning of the experiment than are the animals used for vitamin B tests. The experimental period of a vitamin A test represents a somewhat different phase of the growth curve of the animal than does the experimental period of a vitamin B test. It is beyond the scope of this paper to discuss the factors which might cause the marked differences in the reactions of the vitamin A and vitamin B test animals or to discuss the factors which might contribute to the variability within either group.

In the routine testing of the vitamin content of foods it is often necessary to run preliminary experiments to determine the quantity of vitamin-containing food that will produce a minimum gain in weight. From such preliminary experiments an idea can often be gleaned as to the approximate weight gains that may be expected of the animals on the prescribed dietary regimen. With this information it is possible to determine the number of animals in a group that will be needed in order to prove whether the differences in gain are statistically significant or not. Obviously, it is not economical to feed more animals than are necessary.

If one holds to a strict criterion, reasonable success in making an estimate of the number of animals per lot can be obtained by solving the equation:

$$\frac{\text{MD}}{\sqrt{\underset{-}{2}\,\sigma^2_{X\cdot ACD}}} = 3 \text{ for } n$$

which by elementary algebra becomes:

(d) $$= \frac{18\,\sigma^2_{X\cdot ACD}}{\text{MD}^2}$$

Let us suppose that preliminary experiments have indicated that a mean difference of 25 gm. can be expected between the weight gains of two groups of vitamin A experimental animals in our laboratory.

Substituting in equation (d)

$$n \quad \frac{(18)\ (21.5622)^2}{(25)^2} \quad 13.38$$

and fourteen animals should be assigned to each lot.

## SUMMARY.

A statistical study of the records of 349 vitamin A test animals and 330 vitamin B test animals was made with a view to estimating more accurately the variability of the population of the rats used in this laboratory.

A method of estimating the mean differences in weight gains necessary for significance and of estimating the number of animals per experimental lot is proposed.

Some observations are made as to the mean difference in weight gains and number of animals per experimental lot necessary in order to obtain results of statistical significance in the routine vitamin testing of this laboratory.

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# THE CHEMICAL COMPOSITION OF THE NORMAL AQUEOUS HUMOR OF THE DOG.*

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Modern chemical methods have made possible the quantitative study of the intraocular fluids. With the choice of the dog as the best experimental animal which has a large amount of aqueous humor and which possesses an eye anatomically comparable to the human eye, it was believed that the analysis of the normal aqueous humor obtained from the living dog would serve as a fundamental basis for the further study of the physiological chemistry of the intraocular fluids. This investigation was begun with the point of view that future research might ultimately reveal the relationship between the chemical changes in the aqueous humor and the pathological processes of the ocular diseases.

## HISTORICAL.

For nearly a century, the chemistry of the aqueous humor has been investigated. The values of the chemical constituents have been variable with little or no agreement among the observers, probably because of the differences in technique in obtaining fluid, of the many different methods employed to estimate the chemical compounds, of the physiological state of the intraocular fluid, and of the various kinds of animals used.

The data on the chemistry of the aqueous humor of the dog have been meager. Yudkin (1) found the refractive index of 146 specimens of aqueous humor of the dog to be between 1.33505 and 1.33501. Gala (2) calculated the amount of sodium chloride to be 0.78 per cent, Rados (3) 0.586 to 0.685 per cent, and Mestrezat and Magitot (4) 0.734 per cent. Lehmann and Meesmann (5) estimated a trace to 0.20 per cent protein, Gala (2) 0.048, and Rados (3) a trace to 0.17 per cent. Rados (3) believed the amino acid content to be 0.06 to 0.24 per cent.

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* The expense of this experimentation was defrayed in part by a grant from the Committee on Scientific Research of the American Medical Association.

It is not considered necessary to review the reports of the investigation on other animals such as the ox, horse, pig, and rabbit since Duke-Elder (6) has given recently an excellent survey of the literature in his monograph on the nature of the intraocular fluids.

## EXPERIMENTAL.

To obtain the largest possible amount of aqueous humor the eyes of large, well nourished, healthy dogs which weighed from 15 to 25 kilos and which were fasted for 12 hours were tapped. No dog was used which showed any abnormality of the eye or surrounding structures. The eye fluid obtained from any dog which later developed intraocular hemorrhage was discarded.

The lids were separated by means of a spring wire speculum and the eyeball and cul-de-sac were thoroughly swabbed with warm distilled water. The eye was dried with a cotton swab and 2 drops of a 6 per cent solution of cocaine were dropped upon the eyeball and allowed to remain for 3 minutes. The eye was again washed with distilled water and swabbed dry. The eye was pierced in the upper and outer quadrant at the sclerocorneal junction by means of a No. 26 gauge needle and the aqueous humor withdrawn rapidly by means of a 2 cc. record syringe. It was possible to withdraw from 0.4 to 1.0 cc. from each eye. If the fluid were withdrawn slowly more fluid was obtained but the fluid was not normal aqueous humor but was contaminated with serum. Such fluids were disregarded for chemical analysis.

Ether and amytal anesthesia were used on dogs fasted for 16 hours. The dogs were anesthetized with ether vapor by the cone and bag method. The vapor was given slowly in the beginning of the anesthesia, to avoid struggling. The amytal was injected in doses of 100 mg. per kilo into the peritoneal cavity. It was found that amytal furnished excellent anesthesia for experimental procedures on the eye of the dog. Cocaine was not instilled into the conjunctival sacs.

It was found that a modification of the methods for the analysis of blood constituents could be applied successfully to the aqueous humor available from one eye by the employment of smaller amounts of the solution of reagents in proportion to the amounts of eye fluid and by the application of the micro colorimeter. It was necessary to use 0.2 cc. for the estimation for sodium chloride, phosphate, creatinine, and urea, and 0.5 cc. for amino acid nitrogen, carbon dioxide, and non-protein nitrogen and total nitrogen. The modified methods were checked by the original methods on pooled specimens of eye fluids and on whole blood.

The following method of analysis was used. The Pulfrich-Zeiss refractometer was employed for the determination of the refractive index at 17.5°, the method of Bang (7) for chlorides, of Briggs (8) for phosphates, of Denis (9) for sulfates, of Van Slyke (10) for carbon dioxide, of Pregl (11) for total nitrogen, of Folin and Wu (12) for urea, creatinine, and non-protein nitrogen, of Folin (13) for amino acid nitrogen, and of Benedict (14) for sugar. The amount of protein nitrogen was calculated by the subtraction of the amount of non-protein nitrogen from the total nitrogen.

In a number of dogs which had been dead for 1 to 24 hours it was found that each specimen of aqueous humor gave an abnormally high refractive index. The glucose was observed to be too low to be estimated or absent. However, the total nitrogen and the protein nitrogen had noticeably increased. Human aqueous humor taken shortly after death confirmed the postmortem change of concentration.

## DISCUSSION.

The small amount of aqueous humor available necessarily requires the measurement of minute quantities of the constituents. For this reason many of the previous investigators have taken pooled fluids from eyes obtained from the slaughter house. In this experimental work individual eye fluids of living dogs are utilized to evaluate the normal variations of the concentrations of the major components.

As shown by Tables I to IV, amytal and ether anesthesia produce no effect on the refractive index, creatinine, sodium chloride, non-protein or protein nitrogen content of the aqueous humor of the dog. A comparison of the components of the aqueous (Table IV) humor and the spinal fluid taken from dogs under amytal anesthesia reveals that both fluids have practically the same constitution. The sodium chloride content of the aqueous humor and whole venous blood is higher than that of the spinal fluid. The non-protein nitrogen of the aqueous humor and the spinal fluid is lower in concentration than that of whole venous blood.

TABLE I.

*Composition of Normal Aqueous Humor of Dog.*

| | Low. | High. | Average. | No. of specimens. |
|---|---|---|---|---|
| Refractive index | 1.33478 | 1.33532 | 1.33504 | 34 |
| | *vols. per cent* | *vols. per cent* | *vols. per cent* | |
| Carbon dioxide | 58.3 | 62.5 | 60.2 | 10 |
| | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| Sodium chloride | 540 | 676 | 617 | 35 |
| Inorganic sulfate as S | 0.24 | 0.50 | 0.35 | 15 |
| " phosphate as $P_2O_5$ | 0.83 | 1.5 | 1.1 | 15 |
| Total N | 27 | 55 | 40 | 18 |
| Protein " | 5 | 25 | 15 | 16 |
| Non-protein N | 12 | 40 | 24 | 24 |
| Urea N | 11 | 15 | 12 | 16 |
| Amino acid N | 8 | 10.3 | 9 | 14 |
| Creatinine | 1 | 1.9 | 1.7 | 23 |

TABLE II.

*Analysis of Aqueous Humor under General Anesthesia.*

| Determination. | Anesthesia. | Duration in hrs. | No. of analyses. |
|---|---|---|---|
| Refractive index. | | | |
| 1.33533 | Ether. | ½ | 7 |
| 1.33524 | " | 1 | 9 |
| 1.33513 | " | 2 | 1 |
| 1.33506 | Amytal. | 1 | 6 |
| Creatinine, *mg. per 100 cc.* | | | |
| 1.4 | Ether. | ½ | 2 |
| 1.4 | " | 1 | 3 |
| Sodium chloride, *mg. per 100 cc.* | | | |
| 607 | Ether. | ½ | 1 |
| 631 | " | 1 | 3 |
| 624 | Amytal. | 1 | 5 |
| 613 | " | 2 | 2 |
| Glucose, *mg. per 100 cc.* | | | |
| Before. After. | | | |
| 74 141 | Ether. | ½ | 4 |
| 75 161 | " | 1 | 3 |
| 87 | Amytal. | 1 | 4 |
| 77 | " | 2 | 2 |

## TABLE III.

*Total Nitrogen, Non-Protein Nitrogen, and Protein Nitrogen Content of Aqueous Humor of Dogs under Ether Anesthesia.*

| Dog No. | Volume of aqueous humor. | Total N. | Non-protein N. | Protein N. | Remarks. |
|---|---|---|---|---|---|
| | *cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| 395 | 0.6 | 32 | 16 | 16 | 1 hr. 45 min. anesthesia. Marked conjestion and mild edema of conjunctiva. |
| 396 | 0.6 | 44 | 29 | 15 | 55 min. anesthesia. Marked congestion and mild edema of conjunctiva. |
| 397 | 0.6 | 28 | 22 | 6 | 45 min. anesthesia. Eyes protected. No edema or congestion. |
| 398 | 0.6 | 37 | 19 | 18 | 2 hrs. 15 min. anesthesia. Marked congestion. Severe edema of conjunctiva. |
| 399 | 0.6 | 28 | 17 | 11 | 30 min. anesthesia. Slight edema and congestion of conjunctiva. |

## TABLE IV.

*Comparison of Constituents of Blood, Spinal Fluid, and Aqueous Humor.*

| Dog No. | Fluid. | Refractive index. | Creatinine. | NaCl | Total N. | Non-protein N. | Protein N. | Duration of amytal anesthesia. |
|---|---|---|---|---|---|---|---|---|
| | | | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *hrs.* |
| 522 | Aqueous. | 1.33513 | | 643 | | | | 2 |
| | Blood. | | | 635 | | | | |
| | Spinal fluid. | 1.33513 | | 451 | | | | |
| 565 | Aqueous. | 1.33487 | 1.3 | 595 | 49 | 34 | 15 | 1 |
| | Blood. | | 1.4 | 618 | | 42 | | |
| | Spinal fluid. | 1.33498 | 1.3 | 463 | 45 | 32 | 13 | |
| 566 | Aqueous. | 1.33514 | 1.1 | 635 | 43 | 32 | 11 | 1 |
| | Blood. | | 1.1 | 627 | | 40 | | |
| | Spinal fluid. | 1.33508 | 1.2 | 461 | 46 | 36 | 10 | |
| 567 | Aqueous. | 1.33492 | 1.1 | 618 | 39 | 31 | 8 | 1 |
| | Blood. | | 1.1 | 621 | | 41 | | |
| | Spinal fluid. | 1.33494 | 1.1 | 492 | 37 | 30 | 7 | |
| 569 | Aqueous. | 1.33516 | 1.3 | 632 | 35 | 29 | 6 | 1 |
| | Blood. | | 1.5 | 607 | | 34 | | |
| | Spinal fluid. | 1.33512 | 1.1 | 508 | 36 | 29 | 7 | |
| 570 | Aqueous. | 1.33514 | 1.2 | 643 | 41 | 34 | 7 | 1 |
| | Blood. | | 1.3 | 643 | | 37 | | |
| | Spinal fluid. | 1.33519 | 1.3 | 595 | 43 | 36 | 7 | |

The analyses of the aqueous humor of eyes irritated by the exposure of the conjunctiva and cornea to the ether vapor indicate that there is an influx of serous exudation into the anterior chamber of the eye. The refractive index is increased, but not in proportion to the concentration of protein (Table II). For this reason the refractometric method of the estimation of the protein in fluids is not applicable to aqueous humor. The glucose content is marked by an increase in the eye fluids during ether anesthesia.

The tables show a similarity of the values to those obtained by Duke-Elder (6), Mestrezat and Magitot (4), and Cohen, Killian, and Metzger (15) for the aqueous humor of the ox, pig, and horse and to those reported by Duke-Elder (6) for the special fluid and the dialysates of blood serum. The sodium chloride content is lower than that observed by Gala (2), Lehmann and Meesmann (5), and Mestrezat and Magitot (4).

It is obviously impossible to make other than general comparisons with the data which are in the literature.

## SUMMARY.

The refractive index of normal aqueous humor taken from the eye of the dog under cocaine anesthesia showed a range of values from 1.33478 to 1.33532. The sodium chloride varied from 540 to 676 mg., the inorganic sulfate as sulfur from 0.24 to 0.50 mg., the inorganic phosphates as $P_2O_5$ from 0.83 to 1.5 mg., the carbon dioxide from 58.4 to 62.5 volumes per cent, the total nitrogen from 27 to 55 mg., the protein nitrogen from 5 to 26 mg., the non-protein nitrogen from 12 to 40 mg., the urea nitrogen from 11 to 15 mg., the amino acid nitrogen from 8.2 to 10.3 mg., and the creatinine from 1.0 to 1.9 mg. per 100 cc. of aqueous humor.

Amytal anesthesia produced no change in the concentration of the creatinine, glucose, sodium chloride, total nitrogen, protein nitrogen, non-protein nitrogen or refractive index of the aqueous humor of the dog. Ether anesthesia caused no variation in the amount of creatinine, protein nitrogen, and non-protein nitrogen. The refractive index was slightly, and the glucose content was greatly increased. Local irritation of the eye by ether vapor resulted in an increase of refractive index and protein. The analysis of aqueous humor, spinal fluid, and whole venous blood of the dog under amytal anesthesia showed the concentration of

the sodium chloride was lowest in amount in the spinal fluid and the non-protein nitrogen content was the greatest in the whole venous blood.

We are indebted to Doctors N. B. Berman, H. H. Goldstein, W. W. Bunnell, and P. Fiskio for their assistance on this problem.

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# THE COPPER CONTENT OF INFANT LIVERS.

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That copper is an active factor in hemoglobin production in the rat has, apparently, been demonstrated by Steenbock, Hart, and associates (1), and by McHargue, Healy, and Hill (2). McHargue (3) found more copper in calf liver than in ox liver, while Lindow, Peterson, and Steenbock (4) observed in the whole new born rat a higher percentage of copper than in rats at any other age. Bodansky (5) has shown that the human fetal brain is higher in copper than is the adult brain. The data presented in this paper indicate that the human infant may store copper also in the liver.

Few analyses of the copper content of infant livers have been published. Yagi (6) reported, for a 1 year old infant, a copper content of 28.0 mg. per kilo of fresh organ. Flinn and von Glahn (7) cite a case 9 months old with 3.92 mg., and van Itallie and van Eck (8) four cases, ranging in age from birth to 3 months, with an average of 20.9 mg. per kilo of fresh tissue. The livers of nine children (3 to 12 years) reported by Yagi (6), van Itallie and van Eck (8), Kielholz (9), Schönheimer and Oshima (10), and Flinn and von Glahn (7), contained an average of 9.03 mg. of copper per kilo of fresh tissue.

Data on the copper content of adult livers are considerably more abundant. The results of the analyses of 75 adult livers by Yagi (6), Lehmann (11), Kielholz (9), van Itallie and van Eck (8), Schönheimer and Oshima (10), and Flinn and von Glahn (7) are summarized in Table I. A few other cases reported in the literature have not been included as the data were insufficient for comparative purposes.

Very recently Kleinmann and Klinke (12) have published analyses of the livers of fifteen infants, ranging in age from birth to

2 years. They find an average of 228.1 mg. of copper per kilo of dry tissue. The same authors report for twelve adult livers an average of 27.5 mg. of copper per kilo of dry organ.

## EXPERIMENTAL.

The material analyzed was obtained from cases which were presented for necropsy in the Laboratory of Clinical Pathology. The bodies were received from the hospital and placed in the refrigerator until necropsy was performed. None of the cases was embalmed. As soon as possible after the livers were removed the gallbladders were excised and discarded. The livers were then weighed, cut into small pieces, and placed in Kjeldahl flasks of suitable size. Organic matter was destroyed by digestion with a mixture of nitric and sulfuric acids. The acidity of this solution was adjusted, the iron reduced, and the copper precipitated as the sulfide. This precipitate was dissolved in nitric acid and made up to volume in 100 cc. volumetric flasks. Aliquots of this solution were analyzed according to the method of Biazzo as modified by Elvehjem and Lindow (13). All reagents, including distilled water, were tested and found to be free from copper.

TABLE I.

*Summary of Analyses from Literature of Copper Content of Adult Livers.*

| Author. | No. of cases. | Range of age. | Cu per kilo fresh tissue. | Lowest and highest values. |
|---|---|---|---|---|
| | | *yrs.* | *mg.* | *mg. per kg.* |
| Lehmann | 4 | 33–45 | 3.88 | 2.5 – 5.0 |
| Yagi | 13 | 20–66 | 9.58 | 6.5 –17.5 |
| Kielholz | 4 | 34–76 | 2.77 | 1.52– 3 75 |
| Van Itallie and van Eck | 18 | 21–86 | 8.32 | 3.2 –17.5 |
| Schönheimer and Oshima | 16 | 24–74 | 2.63 | 1.33– 3.92 |
| Flinn and von Glahn | 20 | 25–73 | 4.84 | 2 30–12.42 |
| Total | 75 | 20–86 | 5.86 Average. | 1 33–17.5 |

## DISCUSSION.

Results of the analyses of twenty-five infant livers are given in Table II and for seven adults in Table III. Of the twenty-five infant cases studied seven were of the white, and eighteen of the

## TABLE II.

*Copper Content of Infant Livers.*

| Case No. | Sex. | Race. | Age. | Cu per kilo fresh tissue. |
|---|---|---|---|---|
| | | | | *mg.* |
| 1 | M. | White. | Still-born. | 13.2 |
| 2 | F. | Negro. | " | 25.0 |
| 3 | M. | " | " | 25.1 |
| 4 | " | " | " | 11.8 |
| 5 | F. | " | " | 30.2 |
| 6 | M. | White. | " | 31.9 |
| 7 | " | Negro. | " | 35.8 |
| 8 | F. | " | 27 min. | 14.6 |
| 9 | " | " | 2 hrs. | 29.0 |
| 10 | M. | " | 10 " | 27.6 |
| 11 | " | " | 2 days. | 48.2 |
| 12 | " | " | 4 " | 29.3 |
| 13 | F. | " | 14 " | 57.6 |
| 14 | M. | White. | 28 " | 9.2 |
| 15 | F. | Negro. | 1 mo. | 17.2 |
| 16 | M. | White. | 2 mos. | 19.4 |
| 17 | " | " | 2 " | 21.0 |
| 18 | F. | Negro. | 2 " | 19.6 |
| 19 | M. | " | 3 " | 32.5 |
| 20 | " | " | 3 " | 31.4 |
| 21* | F. | " | 8 " | 6.9 |
| 22 | M. | White. | 16 " | 14.6 |
| 23 | F. | Negro. | 2 yrs. | 20.7 |
| 24 | M. | White. | ? | 11.9 |
| 25 | " | Negro. | ? | 15.5 |
| Average. | | | | 24.0 |

* Severe anemia.

## TABLE III.

*Copper Content of Adult Livers.*

| Case No. | Sex. | Race. | Age. | Cu per kilo fresh tissue. |
|---|---|---|---|---|
| | | | *yrs.* | *mg.* |
| 26 | F. | Negro. | 20 | 5.0 |
| 27 | M. | " | 16 | 3.7 |
| 28 | " | " | 22 | 4.0 |
| 29 | " | " | 37 | 2.6 |
| 30* | " | " | 40 | 2.6 |
| 31 | " | " | 40 | 8.5 |
| 32 | F. | " | 46 | 1.6 |
| Average. | | | | 4.0 |

* Anemia.

negro, race. The average copper content for the whites is 17.3 mg. and for the negroes 26.6 mg. per kilo of fresh tissue. From the numerous uncontrollable factors in a study of this kind, it is impossible to state whether the average difference noted has racial significance. Yagi (6) concludes from the results of his analyses that the copper content of the liver of the Japanese is greater than that of the European.

Case 21, Table II, is of interest in that this lowest copper value for the infants is associated with a severe anemia. With Case 30, Table III, of the adult series, we again have a low copper content associated with anemia.

From our studies, the average copper content of infant liver is 6 times that of the adult. Kleinmann and Klinke (12) find a ratio of approximately 8:1 on the basis of dried liver weight. When we consider that the water content of the liver is larger in the infant than in the adult our ratio is correspondingly increased.

## SUMMARY.

Analyses of the livers of twenty-five infants gave an average of 24.0 mg. of copper per kilo of fresh tissue or 6 times the average value for seven adults.

The highest value for an adult was lower than any of the values for the infants, with the exception of one infant case with severe anemia.

The authors wish to thank Dr. H. C. Schmeisser and Dr. I. H. Jones for assistance in securing the autopsy material used in this investigation.

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# CHEMISTRY OF THE TUBERCLE BACILLUS.

## I. ANALYSIS OF BACILLUS CALMETTE-GUÉRIN (BCG).

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(Received for publication, May 19, 1930.)

The tubercle bacillus, since its isolation by Koch in 1882, has been more intensively studied chemically than any other microorganism. Its high lipid content and ability to form tuberculin have each served as starting points for numerous investigations, while the hope of extracting some substance which might be of therapeutic or diagnostic importance has been a stimulus to many workers.

The experimental work prior to 1900 has been reviewed by Jolles (1), and that from 1900 to 1923 by Wells, DeWitt, and Long (2). The later experiments have been amply summarized by the more recent investigators.

Much of the earlier work has been repeated by investigators who have applied modern and improved methods to the problem of extracting various fractions from tubercle bacilli. So far, these investigators have confined their efforts largely to the human Strain H-37 cultivated on a synthetic medium.

The dissociation of *Bacillus tuberculosis* into virulent S and avirulent R types by Petroff (3) changed the aspect of all bacteriological as well as chemical problems dealing with that organism. The difference in topography and other physical properties of the two types coupled with their difference in pathogenicity, suggested the possibility of chemical differences in their constituents. From this it would follow that the predominance of either type of organism in a given culture, would influence the outcome of an experiment.

It was with the idea of demonstrating differences between these two types by chemical means that the following work was undertaken.

## EXPERIMENTAL.

*Culture.*—The culture employed in this experiment was obtained from the Pasteur Institute, in 1927, and dissociated by Dr. Petroff the same year into R and S types. The two types of apparently pure colonies were then cultivated on Sauton's medium of pH 6.4 for a period of 31 to 36 days, after which the bacilli were removed by filtration, washed, and dried to constant weight *in vacuo* over sulfuric acid. The average yield of organisms was 1.1 gm. of the R and 0.9 gm. of the S type for 100 cc. of culture medium. The reaction of the medium after growth was pH 7.8 and pH 5.6 for the R and S strains respectively.

TABLE I.

*Distribution of Fractions in 50 Gm. of BCG.*

| | R type. | S type. |
|---|---|---|
| | *gm.* | *gm.* |
| Ether-soluble lipid | 11.7 | 5.8 |
| Water-soluble acetic acid-insoluble protein | 0.11 | 0.08 |
| " sodium hydroxide-insoluble protein | 0.25 | 0.07 |
| 5 per cent sodium chloride-soluble | 0.008 | 0.30 |
| 0.5 per cent sodium hydroxide-soluble | 0.85 | 3.78 |
| Glycogen | 0.64 | 2.81 |
| Dialysis precipitate from carbohydrate | 0.09 | 0.46 |
| Insoluble material from carbohydrate | 0.16 | 0.09 |
| Carbohydrate | 5.33 | 4.85 |
| Pooled soluble residue | 1.08 | 1.39 |

The virulence of the two types of bacilli was tested at the time of harvesting by animal inoculation. Animals receiving R type organisms always remained well, while those inoculated with the S type died of generalized tuberculosis.

The procedure employed for isolating the lipid, water-soluble, sodium chloride-soluble, and 0.5 per cent sodium hydroxide-soluble fractions was essentially that of Coghill (4) modified to meet the requirements of the experiment.

In a preliminary study with pulverized bacilli, difficulty was experienced in obtaining clear extracts from the S type by centrifugalization or filtration. To obviate that difficulty in this experiment all extractions were made successively from a single unit of 50 gm. of intact bacilli and continued as long as the fraction sought appeared in the extract.

*Ether Extraction.*—The bacilli were extracted twenty-five times at room temperature with 300 cc. portions of ether (distilled over sodium) during 73 days.

The extractions were interrupted after the fifteenth and a series of six water extractions were made with the hope that the resulting loss of water-soluble material would facilitate the removal of the remaining lipid.

The combined ether extracts were filtered through a Berkefeld filter (N), evaporated at 30° under diminished pressure to a small volume, and to incipient dryness on the hot plate. The final yield of lipid, after drying to constant weight *in vacuo*, was 11.7 gm. from the R and 5.8 gm. from the S bacilli.

*Water Extraction.*—The residues from the ether extraction were extracted ten times during 40 days with water containing a little toluene. The combined R extract when centrifugalized at high speed for ½ hour gave a slightly turbid solution whereas the S extract was still turbid after continuous centrifugalization for 2 weeks. After two filtrations through a Berkefeld filter (N), both extracts were adjusted to pH 6.8 and concentrated under diminished pressure to a volume of 400 cc. The R extract was a brownish yellow, faintly fluorescent solution; the S extract was less chromogenic but more fluorescent.

The protein was precipitated from these solutions with acetic acid and purified by two additional precipitations from alkaline solutions followed by two washings with alcohol and ether. This fraction, when dried *in vacuo* over sulfuric acid, was a nearly white powder yielding 0.11 gm. for the R and 0.08 gm. for the S bacilli.

An alkali-insoluble fraction was obtained from the acetic acid filtrate by the addition of sodium hydroxide. This fraction was a nearly white powder of which 0.25 gm. was recovered from the R and 0.07 gm. from the S filtrates.

*5 Per Cent NaCl Extraction.*—Extraction of the R bacillary residue with 5 per cent sodium chloride was accomplished by two treatments extending over a period of 7 days whereas four treatments, each of 3 days duration, were required to complete the S extraction.

The filtered R extract was a clear colorless solution which yielded upon precipitation with acetic acid 0.008 gm. of protein resembling the water-soluble fraction. Addition of acetic acid to the slightly opalescent S filtrate caused it to become very turbid and prolonged centrifugalization threw down a greenish somewhat gummy precipitate. Although this dissolved readily in dilute sodium hydroxide and became turbid with the addition of acetic acid, no precipitation took place on standing overnight and very little on prolonged centrifugalization.

Inasmuch as this substance was initially precipitated by acetic acid, and on attempts at reprecipitation gave a turbidity, thereby indicating its relationship to the acid-insoluble fraction, it was considered justifiable to recover it by other means and include it in this fraction. Accordingly, it was precipitated with 5 volumes of 95 per cent ethyl alcohol and purified by two further alcohol precipitations from alkaline solution. The final product was a white powder weighing 0.30 gm. Although it bore a resemblance to the carbohydrate fraction, it gave a very weak Molisch test.

*0.5 Per Cent NaOH Extraction.*—After the last saline extraction, the residues were washed with water and extracted nine times, during 25 days, with 0.5 per cent sodium hydroxide.

The R extract, after recentrifugalization and two filtrations through a Berkefeld filter (N), gave a clear, light brown solution. The S extract was still turbid after continuous centrifugalization for 8 days and filtration through the Berkefeld filter requiring 6 days and necessitating a great loss of material. On second filtration, a clear solution a little more chromogenic than the R extract was obtained.

The acetic acid-insoluble material from these extracts was a faintly buff-colored powder of which 0.85 gm. were obtained from the R bacilli and 3.8 gm. from the S type.

*Glycogen.*—The residues from the 0.5 per cent sodium hydroxide treatment were extracted three times for 4 hour periods with 5 per cent sodium hydroxide as described by Johnson and Brown (5) and the acetic acid supernatant solution filtered through a Berkefeld filter.

These extracts when combined with all previously obtained acetic acid filtrates, adjusted to pH 6.8, and concentrated at 45° to a volume of 800 cc., yielded a small amount of precipitate due to the concentration of salts. The R fraction weighed 0.12 gm.; the S fraction was lost due to a broken centrifuge tube.

The concentrated filtrates were treated with hydrochloric acid and alcohol (6) and the precipitate dissolved in dilute alkali. The nucleic acid was separated and discarded and the remaining glycogen-like precipitate purified by the method of Levene (7). The yield of R and S fraction was 0.642 gm. and 2.810 gm. respectively of a perfectly white powder resembling that described by both Levene (7) and Laidlaw and Dudley (8). Both fractions gave no tests for proteins but contained a trace of nitrogen and phosphorus. The S fraction, when hydrolyzed with hydrochloric acid, produced reducing substances equivalent to 98.1 per cent of glycogen, gave a negative reaction with Tollen's reagent and yielded an osazone identical in appearance to glucosazone.

*Carbohydrate.*—The filtrate from the last fraction was concentrated at 40°, dialyzed, and further concentrated to a volume of 200 cc. This caused the formation of a small amount of precipitate, the R fraction of which weighed 0.09 gm. and the S fraction 0.46 gm.

The carbohydrate was precipitated by the method of Mueller (9), dissolved in 60 cc. of water, and freed from a small amount of suspended matter which was insoluble in sodium hydroxide, acetic acid, and hydrochloric acid. The R fraction of this substance weighed 0.16 gm. and the S fraction 0.09 gm. The carbohydrate was again precipitated with alcohol as a practically white powder when in the finely divided state, but as a brown gum when compressed into clumps. The yield from the R bacilli was 5.33 gm. and from the S fraction 4.85 gm.

Portions of these two fractions, further purified by two additional precipitations with alcohol, still gave faintly positive biuret reactions but no other tests for proteins. Hydrolysis with hydrochloric acid produced reducing substances equivalent to 47.0 and 48.3 per cent glucose for the R and S fractions. Both hydrolysates gave positive reactions with Tollen's reagent and produced at least two unidentified crystalline osazones.

*Pooled Soluble Residue.*—The filtrates from the carbohydrate precipitations were combined with all alcohol washings and the alcohol removed by distillation. The concentrated solution was dialyzed salt-free, filtered through a Berkefeld filter (N), concentrated on the water bath at 50°, and evaporated to dryness in a vacuum desiccator over sulfuric acid. Both R and S fractions formed dark brown, glistening deposits which weighed 1.08 gm. and 1.39 gm. respectively.

The distribution of the various fractions is shown in Table I.

## DISCUSSION.

An attempt has been made to demonstrate by chemical analysis differences in the composition of R and S types of BCG. At the beginning of the experiment, it was hoped that the results might be capable of quantitative interpretation, but as the work progressed and the many difficulties in extraction and manipulation were encountered, it was realized that the figures obtained would have more of a relative significance.

Throughout the experiment, beginning with the planting of the bacilli, every effort was made to keep all subsequent procedures identical for both types of bacilli. In this manner, although one type might break down more easily than the other and for that reason yield more readily to extraction, the differences observed would be based on the same initial treatment. This would at least demonstrate differences in behavior of the two types to the same chemical influences although the quantitative yield of fraction would not, due to the difficulties previously mentioned and to losses necessitated by manipulation of highly colloidal solutions (with special reference to the S type) represent the true amount of fraction present in the bacilli.

In spite of the reservations with which these results must be interpreted, several outstanding differences between the two types have been demonstrated. A great difference in physical properties was seen in the manner in which the two types sedimented on centrifugalization. All S extracts were markedly more colloidal than the corresponding R extracts. The differences between the lipid content, sodium chloride-soluble fraction, 0.5 per cent sodium hydroxide-soluble fraction, and the glycogen-like fraction of the two types are too great to be without significance.

## SUMMARY.

1. Under identical chemical treatment, the R or avirulent type produced more lipid than the S or virulent type.

2. The S type gave a greater amount of material, soluble in aqueous inorganic solvents, than the R type.

3. From the above data it is evident that the predominance of either type of organism is a factor which must be taken into consideration when dealing with chemical problems involving bacteria.

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# CHEMISTRY OF THE TUBERCLE BACILLUS.

## II. ANALYSIS OF THE MEDIUM WHICH PRODUCED FIFTY GRAMS OF R AND S TYPE OF BACILLUS CALMETTE-GUÉRIN (BCG).

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(Received for publication, May 19, 1930.)

During recent years the tubercle bacillus, cultivated on some type of protein-free synthetic medium, has been subjected to a program of intensive chemical investigation while comparatively few workers (1–5) have shown an interest in the contents of the medium after growth. From the difficulties encountered in the extraction and manipulation of suspensions of bacilli, it would seem that the medium, in spite of its relatively low protein content, offered a very attractive field for study.

In the preceding paper, differences in the amounts of various fractions extracted from R and S types of BCG were reported and the differences with which the two reacted to the same chemical treatment were emphasized. These differences, inherent in the bacilli, should also be manifested by the products of their metabolism and an analysis of the medium on which they grew should confirm this anticipation.

### EXPERIMENTAL.

The medium which produced the 50 gm. of bacilli reported in the preceding paper gave, after filtration, dark straw-colored slightly fluorescent solutions which were identical in appearance for both types of bacilli. The aqueous washings from the bacilli were combined with their respective filtrates and the whole filtered through a Berkefeld filter, adjusted to pH 6.6, concentrated at 45–50° to approximately 0.1 of their original volume, sterilized at 60° for ½ hour, and stored in the ice box.

The concentrated filtrates were shaken to suspend the small amount of sediment which had formed on standing, 8 volumes of 95 per cent ethyl alcohol (redistilled over sodium hydroxide) were added, and the precipitate removed by centrifugalization. This procedure was so arranged that the total time of contact with alcohol never exceeded ½ hour.

At this point the characteristic odor of Old Tuberculin was noticed in the alcoholic R supernatant solutions but not in those from the S organisms.

The alcohol precipitate was suspended in water, dialyzed, and the portion which remained insoluble after dialysis extracted with water, 5 per cent sodium chloride, and 0.5 per cent sodium hydroxide in a manner similar to that employed in treating the bacilli.

TABLE I.

*Distribution of Fractions in the Medium Which Produced 50 Gm. of BCG.*

| | R type. | S type. |
|---|---|---|
| | *gm.* | *gm.* |
| Water-soluble | 0.06 | 0.08 |
| 5 per cent sodium chloride-soluble | 0.001 | 0.001 |
| 0.5 per cent sodium hydroxide-soluble | 0.08 | 0.37 |
| Insoluble residue | 0.11 | 0.10 |
| " material from glycogen | 0.00 | 0.21 |
| Glycogen | 0.04 | 0.84 |
| Carbohydrate | 0.16 | 1.31 |
| Pooled soluble residue | 0.90 | 3.53 |

*Water Extraction.*—The dialysate and aqueous extract were combined giving yellowish brown R and greenish yellow, slightly fluorescent S solutions from which the protein was removed with acetic acid as described in the preceding paper. The yield of R and S fraction was 0.06 and 0.08 gm. respectively of a white powder.

*5 Per Cent NaCl Extraction.*—The sodium chloride extracts were colorless solutions from which 0.001 gm. of R and S type material was obtained as a nearly white powder.

*0.5 Per Cent NaOH Extraction.*—These extracts were amber-colored solutions which yielded 0.08 and 0.37 gm. of R and S fraction resembling the corresponding fraction isolated from the bacilli.

*Insoluble Residue.*—The insoluble R and S residues from the last extraction were light gray powders weighing 0.11 and 0.10 gm. respectively.

*Glycogen.*—The acetic acid filtrates from all fractions were combined, adjusted to pH 6.8, and concentrated at 45° to a volume of 140 cc. The small amount of R precipitate which formed during the last stages of the concentrating was too small in amount to harvest; the S precipitate weighed 0.21 gm.

The concentrated filtrates were treated in the same manner as the corresponding ones obtained from the bacilli and yielded 0.04 gm. of R type and 0.84 gm. of S material which resembled the glycogen-like fraction of the bacilli and gave faintly positive biuret reactions but no other tests for proteins.

*Carbohydrate.*—The filtrates from the glycogen-like fraction were adjusted to pH 6.9 and the carbohydrate fraction isolated and purified as described in the preceding paper. The yield of R and S fraction was 0.16 and 1.31 gm. respectively of a slightly buff-colored powder which gave a faintly positive biuret reaction. On hydrolysis, the R and S fractions yielded reducing substances equivalent to 53.6 and 50.8 per cent of glucose.

*Pooled Soluble Residue.*—All alcohol supernatant solutions and washings were concentrated by distillation to a volume of 450 cc., evaporated to 140 cc., dialyzed salt-free, and taken to dryness *in vacuo* over sulfuric acid. The end-product was a brownish black gum, the R fraction of which weighed 0.90 gm. and had the characteristic odor of Old Tuberculin. The S fraction weighed 3.53 gm. and did not have this odor.

The distribution of the various fractions in the medium is shown in Table I.

## DISCUSSION.

Differences in the amounts of various soluble substances, elaborated by R and S types of BCG in culture medium during growth, have been demonstrated.

Marked differences are seen in the yields of 0.5 per cent sodium hydroxide-soluble, glycogen and carbohydrate fractions.

In the preceding experiment, a greater yield of soluble constituents, other than lipid, was obtained from the S type of organism. The medium on which the S type grew also gave a greater amount of these substances.

## SUMMARY.

An analysis of the medium on which identical amounts of apparently pure types of BCG were cultivated shows that the S type in the course of its metabolism elaborates a greater amount of soluble substances than the R type.

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# A MICRO COLORIMETRIC METHOD FOR THE QUANTITATIVE ESTIMATION OF IODINE IN BLOOD.

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(Received for publication, June 14, 1930.)

The study of the iodine content of human blood and its relation to disease has been delayed because of the lack of suitable methods for its estimation in small quantities of blood. A method accurate enough and suitable for clinical investigation may prove of value in the early diagnosis of goiter or make clear the relationship of thyroid activity in other diseased conditions.

Various investigators have reported on methods for the estimation of iodine in air, food, water, vegetables, tissue, and blood. Chatin (1) in 1851 used the method of Rabourdin (2) and established the presence of iodine in air. Rabourdin's method depends on converting the iodine to potassium iodide, setting it free with nitrous acid, and extracting with carbon disulfide. Detection was possible in solutions containing 1 part per million of iodine. A year later Grange (3) showed that the starch-iodide reaction could be used in solutions containing 5 parts per million; this later became the basis for the titration method.

In 1913 Blum and Grützner (4) contributed a method for the estimation of iodine in blood. The principle involved combustion of the organic matter by their barium superoxide method, oxidation of iodide to iodate by permanganate, and titration with 0.01 N thiosulfate. The determination required 200 cc. of blood.

Kendall and Richardson (5) in 1920 developed a similar method for the detection of iodine in blood and animal tissue. They oxidized iodide to iodate quantitatively by the action of bromine or chlorine. The iodine is liberated by an excess of potassium iodide. This reaction produces 6 times the amount of iodine originally present. The liberated iodine was then titrated with 0.005 N thiosulfate. The average iodine content of human blood was found to be 0.013 mg. per 100 cc. 100 cc. of blood were required for determination.

Stieglitz (6) in 1923 precipitated iodine as palladous iodide ($PdI_2$) and used this reaction for microchemical detection of iodine in tissues.

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A year later Kelly and Husband (7) modified the method of Kendall, giving the lower limit for their method as 0.005 mg. of iodine.

Von Fellenberg (8) published a method for the estimation of small quantities of iodine. It involves both colorimetric and titrimetric technique. The colorimetric estimation consists of shaking out the liberated iodine with 0.01 cc. of chloroform. With the use of a special magnifying colorimeter the color was matched against a known standard. The colorimetric results were checked by titration with 0.02 N thiosulfate. The lower limit for this method is given as 0.001 mg. of iodine. Veil and Sturm (9) used this method for the estimation of iodine in 10 cc. of blood. They report for human blood an average of 12.8 $\gamma$ per cent (1 $\gamma$ is 0.001 mg.) during summer months and 8.3 $\gamma$ per cent for winter.

Leitch and Henderson (10) modified the method of von Fellenberg by eliminating the colorimetric method and concentrating on the titrimetric method. Titration is made with a 0.1 cc. pipette and requires the accuracy of titrating to 0.002 cc. with 0.02 N thiosulfate. This corresponds to 0.08 $\gamma$ iodine.

Maurer (11) published a method in 1927 for the micro estimation of iodine in blood which he based on the von Fellenberg method. 15 cc. of blood are required. He found the iodine content of venous blood to be 9.2 $\gamma$ per cent.

The following year McClendon (12) introduced his oxygen combustion method for ashing organic material, the use of the Cottrell precipitator in recovering iodine, and his colorimetric method for the estimation of small traces of this element. Colors were matched in a micro colorimeter after shaking out the iodine with 1 cc. of carbon tetrachloride. Accurate results by this method required the presence of 0.01 mg. of iodine, though the limit that may be determined was 0.001 mg.

Attempts were made to determine the iodine content in 10 cc. of blood by the methods of Maurer and Leitch and Henderson. Both methods presented difficulties. Considerable skill is required to titrate accurately to 0.002 cc. Even on known solutions containing 0.001 mg. of iodine consistent results were not obtained by the titration method. The author finds that the color produced by 0.001 mg. of iodine in 0.01 cc. of carbon tetrachloride or chloroform is not detectable by the naked eye. With the use of a magnifying glass distinct differentiation could not always be made between 0.001 and 0.002 mg. of iodine. In order to estimate quantitatively the iodine content in amounts of blood as small as 10 cc. it is necessary that the method be accurate enough to detect a difference of at least 0.0002 mg. Loss of iodine in ashing and extraction for its final estimation must be regarded as the source for greatest error. If the method used for determining the iodine extracted is not sensitive within the above limit, the total error will be such that the method cannot be relied upon.

Blum (13) criticized the colorimetric method of von Fellenberg and the titration method as used by Veil and Sturm. They have shown by experiment that the difference in color produced in 0.01 cc. of chloroform between 0.0005 and 0.001 mg. of iodine is not sufficient to be detected even by the aid of a magnifying glass. Further they state that the color produced is not stable and that the von Fellenberg standard decolorizes on standing. Regarding the titration method, they state that the titration ability of dilute thiosulfate solution begins at 0.004 mg. of iodine and that amounts below this are not detectable. For these reasons they advise their method (1913) which requires the use of 200 cc. of blood.

Owing to the uncertainties and difficulties encountered in the methods thus far described for the micro estimation of iodine in blood, a new method is presented which is believed to be more accurate and is carried out with less technical ability. The estimation of the iodine is based on the color comparison of the starch-iodide reaction, applied after oxidation of iodide to iodate and the liberation of the iodine by an excess of potassium iodide. The depth of color produced in this method by 0.001 mg. of iodine in a volume of 1 cc. is sufficient to be read accurately and easily in a micro colorimeter. The actual amount of iodine liberated to produce the color is 6 times the amount originally present. The method of ashing the blood and extraction of the iodine for the colorimetric estimation is modified considerably from previous methods. The method requires simple apparatus and the ashing is hastened by means of oxygen combustion in an open dish. 90 to 95 per cent recovery from blood is obtainable. In mixtures of starch and potassium iodide equivalent to the iodine content in 10 cc. of normal blood only 85 to 90 per cent could be recovered. With larger amounts (0.1 mg.) the per cent recovered by titration falls to 75 per cent or less.

## *Method.*

### *Reagents.*

4 N solution of potassium carbonate.
Iodine-free water.
Saturated solution of potassium sulfate.
10 per cent solution of barium chloride.
Absolute alcohol.
Saturated bromine water.
2 N sulfuric acid.
1 per cent alcoholic salicylic acid.
1 per cent solution of potassium iodide.
Starch solution (prepared after Nichols (14)).

Standard potassium iodide solution containing 0.001 mg. of iodine per cc.

Potassium oxalate, anticoagulant.

### *Preparation and Purification of Reagents.*

All chemicals used must be tested for traces of iodine and the reagents must be made up with iodine-free water.

Barium chloride, salicylic acid, potassium oxalate, and sulfuric acid were found to be iodine-free. Carbonates and sulfates are freed from iodine by shaking with alcohol and recrystallizing from water. Starch is made iodine-free by successive washing in cold water. The solution is prepared as follows:

Add 0.5 gm. of soluble potato starch to 2.5 cc. of cold water and mix to form a thin paste. Pour it gradually with constant stirring into 200 cc. of water. Boil 15 minutes, stirring continually. Allow to cool and add 0.25 gm. of salicylic acid. Stir until the preservative is dissolved. Fresh starch solution should be prepared every 2 weeks. If a good grade of starch is used, the solution will be clear and filtration unnecessary. Poor grades of starch may produce cloudy solution and filtrates of a faint pink color.

The potassium iodide used for the standard solution and the 1 per cent solution must be purified from traces of iodate by recrystallizing from alcohol and finally from iodine-free water.

Absolute alcohol if found to contain traces of iodine must be redistilled over potassium hydroxide.

Iodine-free water is made by redistilling distilled water from potassium hydroxide. Traps must be used in the distillation to guard against contamination by foreign organic material. Distilled water may be used if it meets the following test.

Evaporate 100 cc. of distilled water to dryness, extract with absolute alcohol, and proceed as directed in the method for the production of the color. If no color is produced it is not necessary to redistil.

### *Preparation of Standard Potassium Iodide Solution.*

1.3081 gm. of recrystallized potassium iodide are weighed out and transferred to a 100 cc. volumetric flask containing 50 cc. of purified water. After dissolving, dilute to 100 cc. (0.1 cc. of this solution is equivalent to 1 mg. of iodine). A weak solution is made by adding 0.1 cc. of the above solution to a liter volumetric flask and diluting to the mark (1 cc. of this solution is equivalent to 0.001 mg. of iodine). Accuracy of the weak solution may be checked by titrating 200 cc. with a 0.005 N thiosulfate solution which has been previously standardized against a 0.005 N solution of potassium iodate.

Take 200 cc. of KI standard, acidify with 2 N sulfuric acid, and add saturated bromine water until the solution is colored orange. Boil off excess bromine and cool. Add a few salicylic acid crystals, 2 cc. of starch solution, and an excess of potassium iodide crystals. Titrate with 0.005 N thiosulfate.

## *Procedure.*

*Ashing.*—0.5 cc. of 4 N $K_2CO_3$ solution (sufficient to make blood alkaline to litmus) is added to a porcelain evaporating dish with a lip (45 cc. capacity). 10 cc. of oxalated blood are introduced by means of a Folin blood pipette. The pipette is rinsed with iodine-free water. The blood should be mixed well by stirring with a small glass rod. The dish with contents is placed in an electric oven set at 110° and the water allowed to evaporate from the blood. This takes 4 to 5 hours, or the dish may be placed in the oven at night and removed in the morning. A crisp, shiny, brown film remains. The dish is then heated moderately over a Bunsen burner until no more fumes come off and the char takes on a black, dull appearance. No more than 15 minutes should be required for this. The bottom of the dish must at no time become more than a dull red. After cooling, the charred blood is powdered with a small porcelain pestle and poured on an open filter paper (15 cm. diameter). The sides of the dish are scraped with the pestle to remove as much of the char as possible. The filter paper containing the char is then folded in the manner used by pharmacists in putting up powders. Carl Schleicher and Schüll filter paper No. 595 has been used for this purpose and found to be iodine-free. The folded paper is set aside for oxygen combustion.

The dish is heated moderately over a Bunsen flame for a minute or so to remove any gaseous material that may be present in the charred particles adhering to the sides. If gaseous material is present small bubbles will be formed on the sides and bottom of the dish. When cool about 5 cc. of iodine-free water are added, the pestle being rinsed off at the same time. The sides of the dish are scraped with a rubber policeman and the contents filtered through a No. 40 Whatman ash-free filter paper, thus removing any unburned carbonaceous material to the filter paper. The filtrate is caught in a 50 cc. centrifuge tube. The dish is rinsed with another 5 cc. portion of water and filtered through the same filter. (All pouring operations must be made quantitatively by pouring down the side of a glass rod.) The dish is then warmed gently to evaporate the last traces of water. The folded filter paper containing the powdered char is placed in the dish with the folded ends upward. They are ignited with a match and the combustion of charred blood and paper hastened by the use of oxygen gas. This is accomplished by attaching the end of a flexible rubber tube to a Hoke-Phoenix regulator which is itself securely fastened to an oxygen gas tank. The other end of the tube is fastened to a short piece of glass tubing (0.5 cm. diameter) bent at right angles. As soon as the ends of filter paper are burned down a steady slow stream of oxygen is allowed to pass through. By means of the glass tip it is directed in a to and fro motion along the inside wall of the dish. The material then burns as a smouldering red mass, burning from the bottom upward. The stream of oxygen is so regulated that the pressure is not sufficient to blow particles of ash out of the dish. As far as possible the material, after the filter paper has burned, should not be allowed to burst into flames. A smouldering red heat is sufficient to burn the blood within 10 minutes. When cool add 5 cc. of iodine-free water. Scrape the sides of the crucible as before and if necessary use a glass rod to loosen all carbonaceous material from the dish. Filter through the same filter paper as before. Rinse the dish with another 5 cc. portion of water, collecting all filtrates in the same 50 cc. centrifuge tube. The filtrate at this time should be clear and at the most a light brown color.

The filter paper with its contents is returned to the original evaporating dish and folded. It is dried and ignited by placing the dish over a Bunsen flame. As soon as the paper is ignited the dish is removed from the flame and the combustion completed by the aid of oxygen, as first described. The remaining ash is extracted with 5 cc. of water, the same procedure as previously described being repeated except that solid particles remaining are allowed to settle and the liquid decanted into the same 50 cc. centrifuge tube by being passed through the funnel without filter paper. The dish with remaining particles is heated over a Bunsen flame at a high temperature until a clean, gray, reddish ash is obtained. Allow this to cool and extract the remaining ash twice with 5 cc. portions of water, powdering all solid particles by pressure with a glass rod. The water extracts are decanted into the centrifuge tube containing previous filtrates and washings. The decantations may produce a slight cloudiness in the combined filtrates. This cloudiness, if there is any, is cleared in the next procedure. The volume of combined washings and filtrates must not exceed 40 cc.

*Extraction.*—To the contents in the 50 cc. centrifuge tube add 2 cc. of saturated solution of $K_2SO_4$ and 5 cc. of 10 per cent $BaI_2$ solution. A white precipitate of $BaSO_4$ is formed. Centrifuge until the supernatant liquid is clear. It should at the most be only a faint yellow color. The supernatant liquid is decanted into a 100 cc. beaker. 10 cc. of iodine-free water are added to the residue remaining in the centrifuge tube. It is thoroughly stirred and loosened from the sides by a small glass rod and centrifuged again, the supernatant liquid being decanted in the beaker containing the first decantation. Repeat the treatment with another 10 cc. portion of water. The contents of the beaker are evaporated to a volume of 5 cc. by boiling gently over a Bunsen flame. The beaker is placed in the oven at 110° to complete the evaporation. The remaining dry residue should be white or only slightly yellow. Remove the beaker from the oven, cool, and extract the contents with absolute alcohol four times. 3 cc. are used for the first extraction; the residue is mascerated and freed from the sides of the beaker with a porcelain spatula. Decant into a 15 cc. centrifuge tube, retaining as much of the residue as possible in the beaker. Repeat the same procedure, using 2 cc. of alcohol for the remaining three extractions. Centrifuge the combined decantations until the alcoholic layer is clear and pour into a 10 cc. platinum crucible. Ignite the alcohol and allow to burn until the crucible is dry. Heat the crucible over a low Bunsen flame with a to and fro motion, the crucible being allowed to glow to a bright red for a few seconds, then remove from the flame until the redness disappears. Repeat this operation until no carbonaceous material remains and only a faint white residue is detectable. Cool and add 1 cc. of iodine-free water. Then rotate the crucible in a slanting position for at least ten complete rotations. The water in the crucible should come in contact with the entire inside surface. Pour into a 15 cc. Pyrex test-tube (125 × 15 mm.) graduated at 1 cc. Add another 1 cc. portion of water to the platinum crucible, rotate as before, and pour the contents into the same graduated test-tube. By bending the top of the crucible together slightly these transfers can be made without appreciable loss of liquid. The solution is set aside for the colorimetric estimation. It should be clear and colorless.

*Colorimetric Estimation.*—A standard containing 0.001 mg. of iodine is made by adding 1 cc. of standard KI solution from a burette to a similar 15 cc. Pyrex test-tube (125 × 15 mm.). 9 cc. of absolute alcohol are placed in a 10 cc. platinum dish and burned as described for the extraction method. After heating, cool and add 1 cc. of water; rotate the crucible and pour the contents into a test-tube containing the standard. Oxidation of iodide is carried out in both standard and unknown. This is based on the method of Leitch and Henderson (10). Add 2 drops (generally sufficient to produce acidity) of 2 N $H_2SO_4$ and 3 drops of freshly prepared saturated bromine water. Let stand $\frac{1}{2}$ minute. Evaporate to a volume of 0.5 cc. by boiling over a low micro burner flame. This must be done cautiously. By holding the tube in a slanting position and continually rotating no spitting or loss of liquid takes place. While still hot add 1 drop of alcoholic salicylic acid and place the tubes in a beaker of cold water. When cooled to room temperature add to each tube 5 drops of starch solution and 3 drops of 1 per cent KI solution and dilute with iodine-free water to the 1 cc. mark. Tubes should not be allowed to stand longer than 20 minutes after oxidation before the addition of starch and KI. A blue color will form immediately if iodine is present. Let stand 5 minutes, dilute the unknown, if necessary, to match standard, and compare in a micro colorimeter. The standard should be set at 20 mm. In the case of very low iodine content the standard must be diluted in order to make a comparison. Unnecessary delay should be avoided throughout the test.

*Calculations.*

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{0.006}{6} \times \frac{100}{10} \times \frac{\text{dilution of unknown}}{\text{dilution of standard}} = \text{mg. iodine}$$

per 100 cc. blood.

As values are expressed in gamma per cent the formula may be made simpler (1 $\gamma$ is 0.001 mg.).

$$\frac{20}{x} \times 10 \times \frac{\text{dilution of unknown}}{\text{dilution of standard}} = \gamma \text{ per cent iodine}$$

For example, with a reading of 15, and with the dilution of the unknown to 2 cc. and 10 cc. of blood, the formula is applied as follows:

$$\frac{20}{15} \times 10 \times \frac{2}{1} = 26\ \gamma \text{ per cent}$$

By allowing blood samples to evaporate overnight six to eight determinations may be completed in one day.

The starch-iodide color produced as given in the method, with quantities of iodine such as are present in 10 cc. of normal blood, may be read in a micro colorimeter with an accuracy of 1 mm. on the scale when compared against a 0.001 mg. standard.[1]

Iodine determinations were made on dog and on human blood. The dog blood was obtained by cardiac puncture. Human blood was drawn from the cubital vein. The values are given in $\gamma$ per cent (1 $\gamma$ represents 0.001 mg.). Of the ten dog blood samples examined only one (from Dog 6) showed an iodine content greater than 15 $\gamma$ per cent. This high value of 39 $\gamma$ per cent may be due to the fact that this dog at the time was nursing four young. Maurer and Diez (15) as well as Blum and Grützner (16) have shown that the iodine content of blood increases during menstruation and pregnancy. If the value for Dog 6 is omitted the average iodine content of blood was found to be 11.1 $\gamma$ per cent.

Known quantities of iodine (0.001 mg.) in the form of KI were added to blood from Dogs 4, 5, 6, and 8 in order to estimate the per cent of iodine recovered. Duplicate determinations were made on the blood of Dog 9. The error in the method as a whole may be considered as 10 to 15 per cent in minute quantities of 0.5 $\gamma$ to 5 $\gamma$. The blank determinations given were carried out identically with the method for blood except that 10 cc. of iodine-free

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[1] Observations on the relative proportionality and stability of the starch-iodide reaction are given in *J. Am. Chem. Soc.* (**52**, 2768 (1930)).

water were substituted in place of blood. The results are recorded in Table I.

The blood findings of nine normal and eleven pathological human blood samples are presented in Table II. The average for normal human blood, 11 $\gamma$ per cent (0.011 mg. per 100 cc.) is slightly lower than that found by Kendall (0.013 mg.). Veil and Sturm (9) find the range for normal human blood to be 8 to 12.8 $\gamma$ per cent. They observed a lower iodine content in winter than in summer. Lunde and Closs (17) give normal blood iodine content in Oslo as 11 to 16 $\gamma$ per cent. The pathological blood samples examined were from cases from the Receiving Hospital and The Shurly Hospital. The first three blood samples were obtained from patients with exophthalmic goiter and the remaining from diabetic cases. The latter were presumably hyperthyroid. Detailed discussion of these results will be given in another paper.

TABLE I.

*Iodine Found in Dog Blood.*

| Dog No. | Blood taken. | Iodine added as KI. | Iodine recovered. | Iodine recovered. | Added iodine recovered. |
|---|---|---|---|---|---|
| | *cc.* | *mg.* | $\gamma$ | $\gamma$ *per cent* | *per cent* |
| 1 | 10 | None. | 1.0 | 10 | |
| 2 | 10 | " | 0.9 | 9 | |
| 3 | 10 | " | 1.0 | 10 | |
| 4 | 10 | " | 0.9 | 9 | |
| 4 | 10 | 0.001 | 1.7 | 17 | 89.4 |
| 5 | 10 | None. | 0.9 | 9 | |
| 5 | 10 | 0.001 | 1.8 | 18 | 94.0 |
| 6* | 10 | None. | 3.9 | 39 | |
| 6 | 10 | 0.001 | 4.5 | 45 | 91.0 |
| 7 | 10 | None. | 1.1 | 11 | |
| 8 | 10 | " | 1.2 | 12 | |
| 8 | 10 | 0.002 | 2.8 | 28 | 87.5 |
| 9 | 10 | None. | 1.6 | 16 | |
| 9 | 10 | " | 1.5 | 15 | |
| 10 | 10 | " | 1.4 | 14 | |
| 10 | 10 | " | 1.4 | 14 | |
| Blank. | 0 | " | 0.0 | 0 | |
| " | 0 | 0.001 | 0.9 | 9 | 90.0 |

$\gamma$ is 0.001 mg.

* Lactation period.

They are given at this time to show that blood samples high in iodine content may readily be distinguished from normal blood by the method presented.

## DISCUSSION.

The use of the starch-iodide reaction for the colorimetric estimation of iodine has been discouraged because of the numerous factors which may alter the color production. McClendon (12) objects to the titration method because of the ease of oxidation of KI. Nichols (14) showed the retarding effect of various salts on the sensitivity of starch reagent. Von Fellenberg (18) attempted colorimetric determination with the iodine-starch reaction and states that in salt solutions it is of little value in quantitative iodine determinations since the intensity is not always proportional to the iodine content. Treadwell (19) as cited by Nichols has

TABLE II.

*Iodine Content of Human Blood.*

| Normal sample. | | Pathological sample. | |
|---|---|---|---|
| Sample. | Iodine. | Sample. | Iodine. |
| | $\gamma$ *per cent* | | $\gamma$ *per cent* |
| Male. | | Female. | |
| A | 9.0 | A | 28.0 |
| B | 9.0 | B | 22.0 |
| C | 9.0 | C | 37.0 |
| D | 10.0 | D | 23.0 |
| D | 10.0 | E | 54.0 |
| E | 11.0 | F | 19.0 |
| Female. | | Mixed, G. | 23.0 |
| F | 13.0 | Male. | |
| G | 12.0 | H | 38.0 |
| H* | 15.0 | I | 29.0 |
| | | J | 31.0 |
| | | K | 19.0 |
| Average......... | 11.0 | | |

The first three pathological samples were obtained from patients with exophthalmic goiter, the remaining from diabetics which were presumably hyperthyroid

* Menstrual period.

shown that starch paste requires the presence of an alkali iodide to prevent the dissociation of the blue iodide starch color. Many investigators have experienced the variability in color from that of a true blue to that of a violet. Nichols (14) prepared a stabilized starch solution which is very sensitive. He eliminates the use of salt or chloroform as a preservative and uses instead salicylic acid. This contribution has aided greatly toward the success of a starch-iodide colorimetric method for minute quantities of iodine.

Conversion of the iodine to $BaI_2$ and extraction with absolute alcohol as given in the method eliminates the interfering effect of other salts, which is the chief cause for unproportional color production with the starch-iodide reaction. Most barium salts with the exception of barium hydroxide are insoluble in absolute alcohol. For this reason $K_2CO_3$ is used to produce alkalinity during combustion. The alcohol used must not impart a blue color to dehydrated $CuSO_4$ in 48 hours.

All glassware must be cleaned with cleaning solution. Traces of organic matter or alkali soaps interfere with the reaction. The bromine must be freshly prepared once a week and always shaken immediately before use to insure complete saturation.

Ashing of the blood was attempted according to the method of Maurer (11) and also in a combustion furnace similar to that described by McClendon (12). The use of the Cottrell precipitator was omitted. McClendon and Remington (20) advised this in samples where the content of iodine determined is low. The method of Maurer did not prove satisfactory as combustion in iron and nickel dishes invariably gave indefinite results because, probably, of the catalytic action of iron in the oxidation of KI. For this reason porcelain dishes are used as containers for the blood during combustion. Platinum-tipped crucible tongs are advised. Even the use of nickle dishes alone proved unreliable. The oxygen method of McClendon was not successful as it was necessary to make three or four combustions before all the organic matter was destroyed. Remington, Culp, and von Kolnitz (21) found that it was unnecessary to follow this laborious technique. They found that 95 per cent of the iodine in sodium iodide when added to potatoes could be recovered after simple ignition of organic matter in a porcelain dish at a temperature which did not exceed 450°. They conclude that organic as well as inorganic iodine is retained by the ash of vegetables when ignited at this temperature. It was found that with the aid of oxygen the simple igniting method required less time. Without the aid of any outside heat, combustion of charred blood is completed within 10 minutes. This is an advantage because the temperature at no time reaches above 450° and a combustion oven can be eliminated. McClendon and coworkers (22) use sulfurous acid to reduce iodates and sodium azide to decompose nitrites in their method for the estimation of iodine in fats. A number of experiments were carried out with sodium bisulfite to reduce iodates but no difference could be detected. Its use has been eliminated as traces of sulfite if carried through oxidize KI in the presence of acid and produce increased color formation. Sodium azide has not been tried, though it is believed that nitrites if formed are reduced to carbonates in the presence of carbonaceous material. Knopheide and Lamb (23) in their method for determination of iodine in minerals remark that nitrates in the presence of charcoal are mainly reduced to carbonate.

The combined water extractions of the ash are pigmented a light brown or yellow. Precipitated barium sulfate practically removes all chromogenic substances without absorption of barium iodide. By this procedure a practically pure alcoholic extract of $BaI_2$ is obtained.

Precautions have been taken throughout the entire method, guarding against loss of iodine through evaporation. Evaporation of water from the blood by direct flame is difficult without loss of material over the side of the crucible. Final evaporation and drying of the water extracts are accomplished in an oven at 110° since loss by spattering is unavoidable when they are attempted over an open flame or a hot plate. Evaporation on a water bath results in condensation of water on the inside of the beaker. The material at this stage must be dry, as traces of water dissolve other salts. Evaporation of the alcohol extract is accomplished by burning the alcohol; other methods tried have resulted in loss of iodine.

In the estimation of so small a quantity of iodine as is present in 10 cc. of blood (approximately 0.001 mg.) it is believed that titration with extreme caution cannot secure as accurate a quantitative iodine determination as a colorimetric comparison in a micro-colorimeter with a 0.001 mg. standard. It is hoped that the method may be applicable to the detection of iodine in small quantities of milk and tissue.

## SUMMARY.

1. A micro colorimetric method is described for the quantitative determination of iodine in small quantities of blood (10 cc.). It involves oxygen combustion in an open dish, removal of interfering substances, oxidation with bromine, and determination of the iodine by comparison of the color produced with the starch-iodide reaction.

2. The method is applicable for the detection of minute amounts of iodine ranging from 0.0005 mg. to 0.005 mg. with an error of 10 to 15 per cent.

This research was carried out at the suggestion of Dr. Burt R. Shurly. The author takes pleasure in extending his appreciation to Dr. Shurly and Dr. R. M. McKean for their cooperation in giving access to hospital patients. The author is also indebted to Mr. C. W. Matthew for his aid in collecting blood for examination.

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# THE ACTION OF BENZOIC PERACID ON SUBSTITUTED GLUCALS.

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The observations to be reported in this communication are the outcome of experiments performed with the aim of converting certain glucose derivatives into the corresponding derivatives of mannose.[1] A convenient method for such transformation was given by Bergmann and Schotte who converted glucal into mannose[2] by the action of benzoic peracid. The reaction proceeded in the same sense in the case of glucals substituted in position (4), 4-glucosidomannose being obtained from cellobial[3] and 4-galactosidomannose[4] from lactal.

*However, we now find that when 3-methylglucal and triacetylglucal are acted upon by benzoic peracid the reaction proceeds abnormally and yields glucose derivatives.* As the glucal is substituted on carbon atom (3) in both of these cases, it seems that the addition of the hydroxyls to the carbon atoms of the glucal is directed by the character of the groups attached to carbon atom (3).[5] Although this abnormal reaction was disappointing for our present purpose, it may be of service on other occasions.

In the case of the triacetylglucal, moreover, the benzoic peracid

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[1] In this connection we may mention that we have obtained from acetobromomannose (from the β-pentacetate) the same triacetylglucal as that from acetobromoglucose, which may be taken to indicate that the two acetobromo derivatives have the same ring structure.

[2] Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, **54**, 440 (1921).

[3] Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, **54**, 1564 (1921).

[4] Bergmann, M., *Ann. Chem.*, **434**, 79 (1923).

[5] It is possible that in the normal reaction an oxide is formed between carbon atoms (2) and (3).

added directly to the double bond and yielded a benzoyl triacetylhexose. The same product was obtained in about equal yield when the reaction was performed in the presence of water. At the moment we are concerned only with the fact that the substance is a derivative of glucose, but we intend to report later on its structure.

## EXPERIMENTAL.

### 3-Methylglucal.

*3-Methyltetracetylglucose.*—This substance was prepared in the usual way by the action of acetic anhydride and pyridine on 3-methylglucose. The product was obtained as a colorless syrup which thus far could not be made to crystallize. It analyzed as follows:

5.860 mg. substance: 10.665 mg. $CO_2$ and 3.215 mg. $H_2O$.

$C_{15}H_{22}O_{10}$. Calculated. C 49.70, H 6.13.

Found. " 49.63, " 6.14.

*Bromo-3-Methyltriacetylglucose.*—This substance was prepared from the tetraacetate with hydrogen bromide in acetic acid in essentially the usual way. Again a colorless syrup was obtained which thus far could not be crystallized. However, its bromine content was practically theoretical; being 19.5 per cent whereas the calculated value is 19.98 per cent.

*3-Methyldiacetylglucal.*—The acetobromo-3-methylglucose was treated with zinc dust[6] in 50 per cent acetic acid in the usual way. Again the product did not crystallize. It was therefore purified by distillation under reduced pressure. At 0.2 to 0.3 mm. pressure it distilled at 125°. In one instance the product obtained in this manner was practically pure glucal on the basis of titration with bromine; in another case, the product titrated for 85 per cent glucal and a second distillation was necessary to obtain an analytically pure product. The yield varied from experiment to experiment. In the best experiment 32.0 gm. of the diacetyl-3-methylglucal were obtained from 100.0 gm. of 3-methylglucose. In another experiment 15.0 gm. were obtained from 60.0 gm. of 3-methylglucose.

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[6] We acknowledge with pleasure our indebtedness to The Sullivan Mining Company for their generosity in furnishing us with zinc dust of high activity.

The substance had the following composition.

5.380 mg. substance: 10.680 mg. $CO_2$ and 3.235 mg. $H_2O$.
$C_{11}H_{16}O_6$. Calculated. C 54.07, H 6.61.
Found. " 54.13. " 6.72.

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-1.32^\circ \times 100}{2 \times 2} = -33.0^\circ \text{ (in chloroform)}.$$

*3-Methylglucal.*—32.0 gm. of the glucal were added to a solution of 80.0 gm. of barium hydroxide and 475 cc. of water, cooled to 0°. The suspension was shaken in a mechanical shaker until the acetylglucal was completely dissolved. Carbon dioxide gas was passed through the solution and the filtrate from the barium carbonate was evaporated to dryness under reduced pressure. The residue was extracted with alcohol and the alcoholic extract concentrated under reduced pressure to a thick syrup which was reevaporated a few times with benzene. Soon the 3-methylglucal began to crystallize on the walls of the flask in long needles. The substance was dissolved in dry ether from which it crystallized again in long needles. It melted at 62–63° (uncorrected) and had the following composition.

4.890 mg. substance: 9.400 mg. $CO_2$ and 3.245 mg. $H_2O$.
$C_7H_{12}O_4$. Calculated. C 52.47, H 7.56.
Found. " 52.42, " 7.42.

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.15^\circ \times 100}{1 \times 1.068} = +14.0^\circ \text{ (in chloroform)}.$$

*Conversion of 3-Methylglucal to 3-Methylglucose.*—Three experiments were performed, the first of which will be described in detail. 5 gm. of the glucal were dissolved in 50 cc. of water chilled to initial ice formation. To the solution were added 5.0 gm. of benzoic peracid dissolved in chloroform. The mixture was shaken until the aqueous layer no longer decolorized a very dilute aqueous solution of bromine. The aqueous layer was then separated in a separatory funnel, extracted several times with benzene to remove benzoic acid, and finally concentrated nearly to dryness and the distillation continued with benzene. The dry residue was taken up in 25 cc. of 99.8 per cent alcohol. 2.0 cc. of this solution were treated with 1 equivalent of phenylhydrazine in the hope of obtaining a phenylhydrazone but none formed. 5.0 cc. were added to an equivalent of benzylphenylhydrazine but again no crystalline hydrazone formed. The remaining solution was concentrated under diminished pressure nearly to dryness whereupon the substance crystallized. The yield was 1.3 gm. For analysis it was recrystallized from ethyl alcohol (99.8 per cent). The substance melted at 160–162° and had the following composition.

5.530 mg. substance: 8.730 mg. $CO_2$ and 3.575 mg. $H_2O$.
$C_7H_{14}O_6$. Calculated. C 43.27, H 7.27.
Found. " 43.05, " 7.23.

The rotation of the substance in water was

| Initial. | Equilibrium. |
|---|---|
| $[\alpha]^{20}_{D} = \frac{+1.90° \times 100}{1 \times 2} = +95.0°.$ | $[\alpha]^{20}_{D} = \frac{+1.05° \times 100}{1 \times 2} = +52.5°.$ |

Thus it was evident that the substance was 3-methylglucose. As Bergmann and his coworkers had used the benzoic peracid in ethylacetate solution, two experiments were performed with this solvent. 3.0 gm. of the glucal were used and the yield of crystalline 3-methylglucose was 1.0 gm. in one case and 1.7 gm. in another.

There remained the possibility that the abnormality of the reaction was due to some impurity in the reagent. To exclude this possibility unsubstituted glucal was acted upon by the same benzoic peracid in both chloroform and in ethylacetate solution and in both cases a nearly theoretical yield of mannose was obtained. The phenylhydrazone formed immediately on addition of the equivalent of phenylhydrazine.

### *Triacetylglucal.*

*Acetoglucal and Benzoic Peracid.*—The acetoglucal was prepared in the usual way and recrystallized from alcohol and petrolic ether, giving a product with a rotation of $[\alpha]^{25}_{D} = -15.1°$ (in 99.8 per cent alcohol).

26 gm. of this acetoglucal were dissolved in 50 cc. of dry chloro-

form and 200 cc. of a 0.54 molal solution of benzoic peracid in chloroform were added (a 10 per cent excess). The mixture was kept at 8–10° for 19 hours and then at room temperature for 5 hours. At the end of this time a sample tested with potassium iodide and acid showed that a little more than the theoretical amount of the benzoic peracid had disappeared. The chloroform solution was shaken repeatedly with sodium bicarbonate solution, washed with water, dried with sodium sulfate, and concentrated under reduced pressure to a syrup. This was taken up in hot isobutyl alcohol from which the product crystallized on standing in the ice box. By concentrating the solution further, two additional crops were obtained. The combined material, 12 gm., was dissolved in 30 cc. of hot isobutyl alcohol and filtered with charcoal. The solution was kept overnight in the ice box, the crystals were filtered off, and washed with petrolic ether-isobutyl alcohol (1:1) and then with petrolic ether. The crystals were fine, colorless needles and the yield of air-dried material was 10 gm. M.p. 139–140°. $[\alpha]_D^{23} = +6.5$ (in chloroform). Further recrystallization gave a material with a m.p. of 140.0–140.5° and

$$[\alpha]_D^{23} = \frac{+0.50° \times 100}{2 \times 4} = +6.2° \text{ (in chloroform).}$$

No mutarotation was observed in 5 hours. The analysis was as follows:

5.495 mg. substance: 11.200 mg. $CO_2$ and 2.590 mg. $H_2O$.
$C_{19}H_{22}O_{10}$. Calculated. C 55.58, H 5.41.
Found. " 55.58, " 5.27.

0.100 gm. of substance shaken with 20.00 cc. of 0.100 N NaOH for 6 hours required 10.10 cc. of 0.100 N HCl for neutralization. Calculated for four acyl groups 9.75 cc. Found, 9.90.

The experiment was repeated but 10 cc. of water were added to the solution of acetoglucal and benzoic peracid in chloroform and the mixture was shaken vigorously in a machine until the reaction was complete (19 hours). The isolation was done in the manner described above. The yield of recrystallized product from 27 gm. of acetoglucal was 9 gm.

The original experiment was repeated, but for the isolation of the product the chloroform solution was concentrated to a thick syrup which was repeatedly extracted with petrolic ether (40–60°). The insoluble residue was taken up in hot isobutyl alcohol. On standing in the ice box, 10 gm. of crystalline product were obtained. After recrystallization from isobutyl alcohol the product melted at 140–141° and had a rotation of

$$[\alpha]_D^{26} = \frac{+0.55° \times 100}{2 \times 4} = +6.9° \text{ (in chloroform).}$$

In order to learn whether the substance was a derivative of mannose or of glucose, 5.0 gm. of recrystallized material were shaken with a saturated (at 0°) solution of dry ammonia gas in dry methyl alcohol, until dissolved. The solution was kept at 2 to 4° for 15 hours and then at room temperature for 4 hours. It was then concentrated under reduced pressure to a thick syrup which was repeatedly extracted with ethyl acetate. The residue was dried, dissolved in water, and diluted to 10.0 cc. A reduction determination (Willstätter) indicated 0.167 gm. of hexose per cc. The rotation, at equilibrium, of 1.0 cc. of the solution diluted to 5.0 cc. with water and measured in a 2 dm. tube (sodium D light) was +3.84°. The rotation, calculated from the reduction value on the assumption that the sugar is glucose, would be $\frac{0.167 \times 52.5° \times 2}{5} = +3.51°$ or if mannose, $\frac{0.167 \times 14.6° \times 2}{5} = +0.98°$.

On treating the above aqueous solution with phenylhydrazine, no insoluble hydrazone was obtained. On heating with phenylhydrazine and acetic acid, glucosazone resulted, being identified by its melting point and mixed melting point with a pure sample.

Thus it seems that the addition product of the acetoglucal and benzoic peracid is a benzoyl triacetylglucose.

# STROPHANTHIN.

## XVIII. ALLOCYMARIN AND ALLOSTROPHANTHIDIN. AN ENZYMATIC ISOMERIZATION OF CYMARIN AND STROPHANTHIDIN.

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In a previous communication (1), the strophanthin which occurs in the seeds of *Strophanthus kombe* has been shown to be a mixture of glucosides of the aglucone strophanthidin. In all of these glucosides, strophanthidin is combined directly with cymarose, a methyl ether $\alpha$-desoxy sugar, as cymarin. This glucoside was found to occur as such in small amount in the mixture but the major portion of the active glucoside was shown to consist of a further combination of cymarin with 1 or more molecules of glucose. By the action upon this mixture of an enzyme which was extracted from strophanthus seeds, it was found possible to hydrolyze all of the glucose linkages without affecting the union between cymarose and strophanthidin, with the result that the complex mixture of glucosides could be converted completely into cymarin. More recently we have attempted to make use of this fact in the course of the isolation from the seeds of the strophanthidin used in our structural studies. It was hoped that by passing through the stage of cymarin it would be possible to accumulate sufficient of the rare sugar, cymarose, for certain future studies. Instead, however, of employing the method already published, which consisted of the preliminary isolation of the crude, amorphous strophanthin mixture and digesting it with a crude enzyme which had also been isolated, a simplification of the process was attempted as follows: The ground and defatted seeds were mixed to a paste with water and after the addition of toluene the mixture was allowed to stand with the hope that the enzyme already present in the seeds would act directly upon the accompanying glucosides. In an examination of the resulting material, observations of a surprizing nature were made. Although the desired enzymatic cleavage of glucose from the complex strophanthins with the production of a simpler glucoside was found to occur, an additional apparently enzymatic transformation was noted, which we believe to be quite unique in character. The extent of this transformation depended upon the duration of the enzyme action. Following the usual procedure, the dilute alcoholic extract of the self-digested seeds after purification with lead acetate and concentration yielded a crystalline substance which proved to be not the expected cymarin. Contrary to cymarin, the new substance was sparingly soluble in chloroform and very soluble in methyl alcohol from which cymarin crystallizes so readily as a methylate. From the analytical results the formula $C_{30}H_{44}O_9$ was derived, so that the new glucoside is isomeric with cymarin. This conclusion was substantiated by the investigation of its hydrolytic products.

The isomerization of cymarin to this substance was found to have occurred in the aglucone portion of the molecule. On hydrolysis the same sugar was obtained which is contained in cymarin, namely cymarose (2). Instead of strophanthidin, however, another substance was obtained, the isomeric character of which was confirmed by the analysis leading to the formula $C_{23}H_{32}O_6$. Contrary to strophanthidin, it was readily obtained from solution in anhydrous form which melted at 248°. We have adopted the designation *allostrophanthidin* for this substance and for the parent glucoside that of *allocymarin.*

Investigation of allostrophanthidin has shown that the oxygen atoms contained in it have retained the functions possessed by those of strophanthidin. Allostrophanthidin still yields an *oxime.* On heating with alcoholic hydrogen chloride it behaves like strophanthidin (3) and is converted into the *ethyl half acetal of oxidodianhydroallostrophanthidin,* which is isomeric with the analogous derivative of strophanthidin. From these facts the aldehyde group is retained in this substance as well as the two tertiary hydroxyl groups which are removed as water to form the dianhydro derivative, and finally the secondary hydroxyl which participates in the oxidic linkage in the above half acetal. As in strophanthidin, the remaining 2 oxygen atoms belong to the lactone group. Allostrophanthidin still gives the nitroprusside reaction characteristic of the $\Delta^{\beta,\gamma}$-unsaturated lactones. In agreement with this, *dihydroallostrophanthidin* was obtained on hydrogenation. The attempt was made to prepare the usual iso derivative with methyl alcoholic alkali. A number of different procedures were tried and although a reaction was found to occur in each case no tangible crystalline substance could be isolated and the major reaction product proved to be always of amorphous acid character.

Since the transformation of strophanthidin into the new isomer has not involved a change of function of the characteristic groups of the former, this isomerization appears to be of purely stereochemical character. The above failure to obtain an isoallostrophanthidin with methyl alcoholic alkali suggests that a *cis-trans* reallocation has occurred of the groups participating in this reaction or of groups which may influence the reactivity of such participating groups. It was hoped that such an explanation might be checked by passing to trianhydroallostrophanthidin which might prove identical with trianhydrostrophanthidin, a benzenoid derivative. For this purpose, the ethylal of dianhydroallostrophanthidin was treated with concentrated hydrochloric acid. Instead, however, of the formation of the trianhydro derivative such as is produced in the case of strophanthidin, a chloro derivative was obtained. Since difficulty was experienced in obtaining the expected analytical figures with this substance it was not further investigated. For the present the exact relationship of allostrophanthidin to the parent strophanthidin remains undetermined.

The above transformation is apparently of enzymatic character. If the paste of kombe seeds was first heated to destroy the enzymes and was then allowed to stand, on working up the mixture only strophanthidin could be obtained on hydrolysis of the crude glucosides. In a former communication a crude enzyme, "strophanthobiase," was described (1), which had been employed for the cleavage of all of the glucosides into cymarin. At that time only cymarin was noted and no suspicion was obtained of the formation of any such substance as allocymarin. More recent experiments have confirmed these results. When a crude enzyme was prepared from kombe seeds in the usual manner and was precipitated by alcohol or acetone, such an enzyme when allowed to act for a long time on the crude glucoside mixture caused cleavage to cymarin only and no appreciable formation of allocymarin was noted. Either the effective isomerizing enzyme is not extracted by the method employed or it is very labile and easily inactivated by acetone or alcohol. Although it is probable that a proper procedure may be found for the separation of such an enzyme from the seeds, we have not for the present made further attempts in this direction. If the above conclusion is correct that we are dealing with a stereochemical rearrangement produced by an enzyme, the observation is of considerable interest. The only previous reference which we have been able to find concerning a stereochemical enzymatic transformation is the so called "Waldenase" of Fränkel (4). To such an enzyme this worker has referred the transformation of the usual *l*-tyrosine formed on tryptic digestion of casein to *d*-tyrosine through *d*-tyrosine anhydride on the prolonged action of trypsin.

Before the above work was begun we had accumulated in the course of the preparation of strophanthidin the cymarin which occurs as such in *Strophanthus kombe* seeds. This was secured as previously described by the extraction with chloroform of the purified alcohol-free solution of the crude glucosides and precipitation of this chloroform solution of crude cymarin with ligroin. Cymarin was then obtained as the methylate by recrystallization from methyl alcohol. In the final methyl alcoholic mother liquors considerable material remained and the attempt was made to repeat the chloroform extraction of this material for purification. A relatively small portion of a crystalline substance appeared at this point, which was very sparingly soluble in chloroform. After our experience with allocymarin we turned again to an investigation of this substance and found it to be identical with allocymarin. The latter, therefore, occurs as such in very small amounts in the seeds, a fact which is not at all strange in view of the artificial transformation described above. A small amount of such a transformation can quite conceivably occur in the seeds themselves.

An investigation of the pharmacological action of the new glucoside has also led to a surprizing result. It has been found to be practically devoid of the characteristic digitalis action in amounts which could be satisfactorily injected into frogs. The latter were found to tolerate as much as 2 mg. in a 35 gm. frog. Unfortunately the sparing solubility of the substance made it difficult to reach the toxic limit of the substance. 4 mg. of the substance in suspension in dilute alcohol failed to kill a 25 gm. frog. For such an animal 0.015 mg. of cymarin sufficed. If the chemical difference between cymarin and allocymarin is merely stereochemical, this difference in pharmacological action is of considerable interest.

Finally, in hydrogenation experiments with allostrophanthidin with the platinum oxide catalyst of Adams and Shriner, it was found that the reaction may be carried beyond the 1 mol stage which gives rise to the dihydro derivative described above. A second mol of hydrogen is more slowly absorbed with the formation of a neutral substance, *dihydroallostrophanthidol.* The second reaction was found to consist in the reduction of the aldehyde group to the primary alcoholic group. Since in our former experience with the catalytic hydrogenation of strophanthidin itself the platinum oxide catalyst had not been employed and such a reaction had not been noted, we have investigated the behavior of strophanthidin towards this reagent. 1 mol of hydrogen was fairly readily absorbed but apparently more slowly than in the case of allostrophanthidin and under very careful conditions it was found possible to introduce an additional mol of hydrogen with the formation of the dihydro alcohol, *dihydrostrophanthidol.*

## EXPERIMENTAL.

1500 gm. of *Strophanthus kombe* seeds which gave uniformly the green color with 80 per cent sulfuric acid were coarsely ground in a meat grinder and were defatted with gasoline. The dried powder was mixed with 3500 cc. of water and 100 cc. of toluene and the mixture was allowed to stand at 25° for 13 days. The mass was then treated with 12 liters of 95 per cent alcohol. After standing 24 hours the mixture was filtered. The filtrate was cleared as usual with lead acetate solution and the excess lead was removed with $H_2S$. During the concentration under diminished pressure in order to remove the alcohol, a copious crystallization occurred. At this point it was found advisable to centrifuge the precipitate and continue the concentration of the mother liquor until the alcohol was completely removed. The remaining aqueous solution was recombined with the crystalline fraction and the mixture was shaken with 200 cc. of chloroform to dissolve any cymarin which had escaped transformation. After centrifuging in order to separate the chloroform layer, the extraction with fresh chloroform was repeated. From the combined chloroform extracts, after shaking out with dilute sodium carbonate solution, concentration and precipitation with petrolic ether, 17 gm. of crude cymarin were recovered. This readily crystallized from methyl alcohol.

*Allocymarin.*—The above crystalline residue undissolved by the chloroform was collected by suction. After recrystallization from 95 per cent alcohol the glucoside formed globules of minute crystals which after collecting with alcohol and drying weighed 33 gm. When recrystallized by careful dilution of the hot alcoholic solution it formed long, narrow, thin leaflets which softened above 145° and slowly effervesced at about 150°.

Allocymarin is easily soluble in methyl alcohol and acetone, is less readily soluble in ethyl alcohol and practically insoluble in water. Contrary to the extremely bitter cymarin, it is practically tasteless.

$[\alpha]_D^{20} = +43$ (c = 1.007 in methyl alcohol).

For analysis the substance was dried at 100° and 15 mm.

4.523 mg. air-dry substance: 0.265 mg. $H_2O$.
$C_{30}H_{44}O_9 \cdot 2H_2O$. Calculated. $H_2O$ 6.16. Found. $H_2O$ 5.85.
4.258 mg. substance: 3.140 mg. $H_2O$, 10.210 mg. $CO_2$.
5.690 " " : 2.125 " AgI.
$C_{30}H_{44}O_9$. Calculated. C 65.66, H 8.09, $OCH_3$ 5.65.
Found. " 65.39, " 8.25, " 4.93.

Allocymarin was obtained also by direct alcoholic extraction of the seeds without previous digestion. This was found in the mother liquors which had accumulated from the recrystallization from methyl alcohol of the cymarin fraction of the crude glucoside mixture. From about 200 pounds of such seeds about 15 gm. of allocymarin were obtained. It separated from 95 per cent alcohol in characteristic narrow, thin platelets which melted with slow effervescence at 150° after preliminary softening and agreed in all properties with the allocymarin prepared by digestion of the seeds.

$[\alpha]_D^{20} = +42$ (c = 0.970 in methyl alcohol).

The identity was confirmed by hydrolysis to allostrophanthidin and cymarose.

5.098 mg. air-dry substance: 0.323 mg. $H_2O$.
$C_{30}H_{44}O_9 \cdot 2H_2O$. Calculated. $H_2O$ 6.16. Found. $H_2O$ 6.33.
4.775 mg. substance: 3.520 mg. $H_2O$, 11.492 mg. $CO_2$.
$C_{30}H_{44}O_9$. Calculated. C 65.66, H 8.09.
Found. " 65.63, " 8.25.

The following experiment was made in order to obtain more conclusive evidence of the enzymatic origin of the transformation of the strophanthin glucosides into allocymarin.

500 gm. of kombe seeds after grinding and defatting yielded 350 gm. of dry powder. Separate portions of this material were treated differently as follows:

150 gm. of the seed powder were extracted three times with 700 cc. portions of 70 per cent alcohol. The combined extract was purified in the usual way with lead acetate and after removal of excess lead with $H_2S$ the alcohol was removed under diminished pressure. The concentrated aqueous solution was cautiously acidified with HCl until slightly acid to Congo red paper. The mixture was then hydrolyzed at 70°. Characteristic rhombs and compact prisms of strophanthidin separated. After filtration a second crop was obtained from the filtrate by the addition of ammonium sulfate. After recrystallization each of these fractions melted at 170° and exhibited all of the properties of strophanthidin. The combined weight was 4.9 gm.

150 gm. of the seed powder were mixed with 450 cc. of water and 50 cc. of toluene and after digesting at 25° for 11 days the mixture was treated with 1400 cc. of alcohol. The alcoholic extract was worked up in the usual manner. In this case on concentration of the purified extract the glucoside crystallized as described above. The acidified aqueous suspension was heated at 70°. The sparingly soluble glucoside slowly dissolved and was soon replaced by long prisms and tables of allostrophanthidin. After recrystallization from alcohol 4.2 gm. of crystals melting at 247° were obtained. A second fraction which was recovered from the aqueous mother liquor of the first fraction by salting out with ammonium sulfate gave after recrystallization 1.6 gm. which melted at 240°.

A parallel experiment was conducted after destruction of enzymes as follows: 50 gm. of the seed powder were treated with 170 cc. of boiling water and the mixture was kept above 90° for 10 minutes. After cooling, 30 cc. of toluene were added and the mixture was kept alongside of the above experiment at 25° for 11 days. On hydrolysis of the resulting glucoside mixture only strophanthidin was obtained.

### *Hydrolysis of Allocymarin.*

*Allostrophanthidin.*—5 gm. of allocymarin were treated with a mixture of 35 cc. of alcohol, 25 cc. of $H_2O$, and 12 cc. of HCl (1.19). The resulting suspension was shaken at 20–25° for 24 hours. This time was required for solution and hydrolysis of the sparingly soluble glucoside. After dilution allostrophanthidin separated very incompletely. On partial concentration of the mother liquor to remove the alcohol most of the dissolved aglucone was recovered, although a small portion still remained in the aqueous sugar solution. This was recovered in the process of isolating the sugar as given below.

On recrystallization from 95 per cent alcohol allostrophanthidin separates as rather sparingly soluble prisms or plates which are anhydrous and melt at 248–250°, with slow effervescence. This melting point was found with the freshly recrystallized substance. In some cases after months of standing in the dry state the melting point was found to increase, and in one case 274° was noted. The substance is practically tasteless. It is sparingly soluble in the usual neutral solvents. It is more readily soluble in the somewhat diluted water-miscible solvents. It gives the characteristic nitroprusside test. The brown color which develops in sulfuric acid is indistinguishable from that produced by strophanthidin.

$[\alpha]_D^{20} = +37$ (c = 0.508 in 95 per cent alcohol).

3.815 mg. substance: 2.815 mg. $H_2O$, 9.577 mg. $CO_2$.
$C_{23}H_{32}O_6$. Calculated. C 68.27, H 7.98.
Found. " 68.45, " 8.25.

*Cymarose.*—The above aqueous filtrate from allostrophanthidin was treated with excess silver carbonate. The filtrate from silver chloride was treated with $H_2S$ and then concentrated to small bulk under diminished pressure. A small additional deposition of the aglucone occurred during this process. The filtrate was then concentrated to dryness. The residue was extracted with dry ether and the extract was allowed to stand over fused $Na_2SO_4$ for several days. On being treated with petrolic ether, the concentrated ether solution gradually crystallized, when seeded with cymarose, as delicate needles. After recrystallization and long drying in a desiccator over $CaCl_2$ the sugar melted at 93°. This melting point, however, was obtained only with carefully dried material. When first collected or on exposure to the air small amounts of solvent or moisture which cling to the substance easily depress its melting point.

The sugar exhibited only slight mutarotation in aqueous solution. For the final reading

$[\alpha]_D^{20} = +52$ (c = 2.01 in $H_2O$).

3.610 mg. substance: 2.840 mg. $H_2O$, 6.880 mg. $CO_2$.
$C_7H_{14}O_4$. Calculated. C 51.81, H 8.70.
Found. " 51.98, " 8.80.

*Allostrophanthidin Oxime.*—This was prepared in the usual manner from the aglucone and hydroxylamine in alcoholic solution. It formed small four-sided tables and prisms from alcohol which effervesced at 235° after preliminary softening. For the analysis it was found necessary to dry the substance in the micro dryer at 120°.

4.536 mg. substance: 3.285 mg. $H_2O$, 10.925 mg. $CO_2$.
5.100 " " : 0.158 cc. N (772.6 mm., 28°).
$C_{23}H_{33}O_6N$. Calculated. C 65.83, H 7.93, N 3.34.
Found. " 65.69, " 8.09.
" N 3.57.

*Dihydroallostrophanthidin.*—Allostrophanthidin was hydrogenated in alcoholic solution with the platinum oxide catalyst of Adams and Shriner. Within 20 minutes approximately the equivalent of 1 mol of $H_2$ was absorbed and the operation was interrupted at this point. The dihydro derivative crystallized from dilute alcohol as long, narrow platelets which softened at 150° as if giving off solvent and finally melted and effervesced at 230°. It is easily soluble in alcohol and acetone and but sparingly so in chloroform. It does not give the nitroprusside reaction.

For analysis the substance was dried at 100° and 15 mm. over $H_2SO_4$.

4.365 mg. substance: 3.370 mg. $H_2O$, 10.920 mg. $CO_2$.
$C_{23}H_{34}O_6$. Calculated. C 67.99, H 8.44.
Found. " 68.22, " 8.64.

*Dihydroallostrophanthidol.*—When the above hydrogenation was carried further, an additional mol of $H_2$ was absorbed more gradually. The reaction was interrupted after 18 hours. The dihydro alcohol separated from its concentrated alcoholic solution as lustrous leaflets which melted at 222° after preliminary softening. The substance did not react with hydroxylamine.

For analysis the substance was dried at 100° and 15 mm. over $H_2SO_4$.

4.603 mg. substance: 3.650 mg. $H_2O$, 11.465 mg. $CO_2$.
$C_{23}H_{36}O_6$. Calculated. C 67.60, H 8.89.
Found. " 67.92, " 8.88.

*Ethyl Half Acetal of Oxidodianhydroallostrophanthidin.*—Allostrophanthidin was refluxed for 30 minutes in 10 parts of 5 per cent absolute alcoholic hydrochloric acid. On cooling needles of the ethylal separated.

After recrystallization from alcohol it melted at 191°.

$[\alpha]_D^{25} = -63$ (c = 1.01 in chloroform).

4.280 mg. substance: 3.135 mg. $H_2O$, 11.870 mg. $CO_2$.
4.302 " " : 2.430 " AgI.
$C_{25}H_{32}O_4$. Calculated. C 75.71, H 8.14, $OC_2H_5$ 11.35.
Found. " 75.64, " 8.20.
" $OC_2H_5$ 10.82.

*Dihydrostrophanthidol.*—Strophanthidin which had been repeatedly recrystallized from glass-distilled alcohol was hydrogenated as described in the case of allostrophanthidin. Within 12 minutes 1 mol of $H_2$ was absorbed. When interrupted at this point dihydrostrophanthidin was obtained. After 18 hours an additional equivalent of $H_2$ was absorbed. The filtrate from the catalyst crystallized on concentration and dilution. It formed needles and prisms from 95 per cent alcohol which melted slowly at 160–163° after preliminary sintering. Its melting point was definitely depressed on mixing with dihydrostrophanthidin and it is also less soluble in alcohol than the latter.

4.640 mg. substance: 3.760 mg. $H_2O$, 11.500 mg. $CO_2$.
$C_{23}H_{36}O_6$. Calculated. C 67.60, H 8.89.
Found. " 67.60, " 9.07.

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# THE DIGITALIS GLUCOSIDES.

## V. THE OXIDATION AND ISOMERIZATION OF GITOXIGENIN.

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Certain abnormalities in the behavior of isogitoxigenin and its derivatives as contrasted with that of isodigitoxigenin and other "iso genins" have repeatedly appeared in our studies with these substances and have been discussed in previous reports (1, 2). The outstanding differences were the stability of the lactol form of isogitoxigeninic acid which results on saponification of isogitoxigenin, and the relative stability of the lactone group in isogitoxigenic acid which results from the oxidation of this lactol group in the former acid. In the work which has led to the conclusion that gitoxigenin is hydroxydigitoxigenin (2), it was shown that the remaining free hydroxyl group in isogitoxigenic acid can be removed to form the anhydro acid. This anhydro acid, in contrast with the saturated parent acid, could be easily saponified by 0.1 N alkali. In addition it was readily hydrogenated with simultaneous cleavage of the lactone group to the desoxy acid, digitoxanoldiacid. Since the removal of this hydroxyl group influenced so greatly the stability of the lactone group, the conclusion was reached that this hydroxyl group must be situated on a carbon atom either within or adjoining the lactone ring. At the time it was assumed that in the oxidic ring of isogitoxigenin the same tertiary hydroxyl was involved as that which forms the oxidic ring of isodigitoxigenin. More recent observations have shown that this last assumption is not true and that different hydroxyl groups participate in the cyclization in each of these iso compounds. In the case of isodigitoxigenin this has been definitely shown to be the tertiary hydroxyl group (3). In isogitoxigenin this group has now been found to be the additional hydroxyl by which gitoxigenin differs from digitoxigenin. This was made evident by the fact that whereas gitoxigenin forms a dibenzoate (4) and digitoxigenin forms a monobenzoate isogitoxigenin on acylation yields only a monoacyl derivative, *isogitoxigenin benzoate.* The additional acyl group in gitoxigenin dibenzoate must be situated on the extra hydroxyl group which is not present in digitoxigenin and forms the oxidic ring of isogitoxigenin. This conclusion was confirmed by the study of the oxidation products of these substances.

The formation of isogitoxigenon on oxidation of isogitoxigenin with chromic acid has already been described (5). The production of a monoketone is in agreement with the assumed presence of one secondary hydroxyl group. This has been noted also in the case of digitoxigenin and isodigitoxigenin (6). On reexamining the so called gitoxigenon which was briefly described in a previous communication (7), observations have been made which do not agree with its provisional characterization as a monoketone. The originally published analytical figures did not agree so well with the required formula, $C_{23}H_{32}O_5$, but rather with the alternative formula $C_{23}H_{30}O_5$, also presented. Recent work has shown that in the formation of this substance two hydroxyl groups have been oxidized to carbonyl so that it is a dioxo derivative, $C_{23}H_{30}O_5$. It is, therefore, obvious that the extra hydroxyl group of gitoxigenin is not tertiary but is either of secondary or primary nature. The fact that the above isogitoxigenin forms only a monoketone and not a dioxo derivative is in agreement with the acylation results in determining the identity of the hydroxyl group involved in the oxidic ring of isogitoxigenin.

A further study of this oxidation product of gitoxigenin has led to the surprizing result that it no longer possesses the unsaturated lactone group of gitoxigenin. It does not give the nitroprusside reaction. Digitoxigenon, which was examined for comparison, was found to give the expected color reaction, a result in agreement with the fact that it can be isomerized by alkali to isodigitoxigenon (6). The explanation of the behavior of the dioxo compound from gitoxigenin was found in the fact that it is an iso compound. The oxidation of the extra hydroxyl group of gitoxigenin to carbonyl has induced a simultaneous isomerization of the type found in isodigitoxigenin in which the double bond is replaced by an oxidic bridge with the remaining tertiary hydroxyl. This was confirmed by the fact that it was not possible to convert the dioxo compound into an anhydro derivative by the action of mineral acid. As presented further on, such dehydration may be produced in the dioxo derivative of dihydrogitoxigenin in which isomerization to an iso derivative is no longer possible. The oxidation product of gitoxigenin is, therefore, a derivative of a normal isogitoxigenin which is isomeric with the isogitoxigenin formed by the alkali isomerization of the aglucone.

Further study of this oxidation product has brought to light an additional complication. It is a mixture of two isomers when prepared by the method originally given in which the chromic acid solution employed contained sulfuric acid. This was caused by an isomerization of the primary product of the reaction under the influence of acid. When the oxidation was accomplished in acetic acid solution with aqueous chromic acid, the principal product was a neutral dioxo derivative which showed a specific rotation of $[\alpha] = +88$. When this substance was allowed to stand with dilute mineral acid isomerization occurred with the formation of a substance possessing a rotation of $[\alpha] = +166$.

In view of the direct relationship of these substances to isodigitoxigenon, the first of these isomers has been at least provisionally designated as α-(*oxoisodigitoxigenon*) and the latter as β-(*oxoisodigitoxigenon*). When the α compound was gently saponified with dilute alkali and then relactonized by gentle acidification with acetic acid, isomerization also occurred since the substance recovered proved to be the above β compound. In an attempt to recrystallize the oxidation product from methyl alcohol it was found to yield a more sparingly soluble substance of higher melting point. This substance proved to contain methoxyl. Under the conditions described in the experimental part, either isomer was found to yield the same substance which proved to be a dimethyl acetal. The methoxyl groups were stable to alkali but were readily removed by dilute acid. This at first suggested the possibility that the extra carbonyl group is of aldehydic character. But a parallel experiment with isogitoxigenon showed that this substance also yields a dimethyl acetal with analogous properties. This is then a property of the carbonyl group common to both substances and which is in turn formed from the secondary alcoholic group common to both gitoxigenin and digitoxigenin. Such ready formation of a ketone acetal by simple heating with methyl alcohol is unusual, since it is a property usually credited to reactive aldehyde groups.

In the hope of shedding additional light on the mechanism of the formation and the properties of these isomeric dioxo compounds, we have turned to an analogous study of the oxidation of dihydrogitoxigenin where any simultaneous oxidation to the above type of iso derivative would be excluded, and in which the tertiary hydroxyl group would be retained. By using a chromic acid solution without the addition of sulfuric acid, the dihydrogenin yielded both a neutral fraction and an acid fraction. The neutral product proved to be mostly a dicarbonyl derivative, $C_{23}H_{32}O_5$, which possessed a specific rotation of $[\alpha] = +89$, and which we have called *α-(oxodihydrodigitoxigenon)*. When this substance was treated with mineral acid it was readily converted into an anhydro derivative, *anhydro-β-(oxodihydrodigitoxigenon)*. There was probably simultaneous isomerization to the β series. The rotation of the latter was found to be $[\alpha] = +154$. The dehydration appeared to keep pace with the apparent isomerization so that it was not possible to intercept any intermediate β form before such conversion into its anhydro form had occurred. When the above α-oxidation product was gently saponified and then relactonized by gentle acidification with acetic acid, the substance which crystallized proved to be also an anhydro compound but apparently still different from the above anhydro derivative. It appears that still another type of isomerization has occurred under the influence of alkali. This second anhydro compound was formed also by saponification and relactonization of the above anhydro-β-(oxodihydrodigitoxigenon).

The lability of the tertiary hydroxyl group with consequent anhydro formation may be conditioned by a *trans-cis* rearrangement of the hydrogen atom and hydroxyl group which are removed as water. The additional type of isomerization under the influence of alkali may be due to a further shift of the double bond. The loss of water during saponification is apparently caused by the influence of the extra carbonyl group. This was indicated by our experience with dihydrodigitoxigenin which was run as a control. This substance was readily converted into *dihydrodigitoxigenon*. When the latter was saponified and then gently acidified with acetic acid, as in the case of the gitoxigenin derivative, α-(oxodihydrodigitoxigenon), dehydration with the formation of an anhydro derivative did not occur and dihydrodigitoxigenon was recovered.

Another substance of interest was isolated from the acid fraction of the oxidation mixture obtained from dihydrogitoxigenin. This crystalline acid proved to be a constant by-product of the action of $CrO_3$ in the presence or absence of sulfuric acid and under different temperature conditions. From the analytical results the formula $C_{23}H_{32}O_6$ was derived for this substance. Titration showed the presence in it of one carboxyl group as well as its molecular size. This was confirmed by the analysis of the *methyl ester*. However, on heating even with 4 per cent alkali at 130° it was not possible to detect the consumption of the extra equivalent of alkali required by the lactone group originally present in the parent substance. Repeated experiments on attempted saponification with 0.1 N or N alkali gave the same result. On back titration in every case, however, it was found that about 10 to 15 per cent of 1 equivalent of alkali was nevertheless consumed in addition to that required for direct neutralization of the free acid group. A reason for this is difficult to picture and we are convinced of the homogeneity of this acid oxidation product. Contrary to the neutral dioxo derivative, the acid could not be converted into an anhydro acid by solution in concentrated hydrochloric acid. The neutral dioxo derivative is not an intermediate stage in the formation of this acid since the latter was not produced in an attempted reoxidation of the former.

The difficulty of procuring the rare and costly material needed in this work has made it necessary for the moment to interrupt further investigation of these substances in directions which are naturally suggested. The behavior of gitoxigenin and its dihydro derivative on oxidation, which has just been presented, has definitely shown that the additional hydroxyl is not of tertiary character as originally assumed and that it is situated on a carbon atom which is most likely δ to the γ-carbon atom of the lactone group of gitoxigenin.

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## EXPERIMENTAL.

### *Oxidation of Gitoxigenin.*

*α-Dioxo Derivative* (*α-(Oxoisodigitoxigenon)*).—The procedure used for the oxidation with chromic acid differs somewhat from that previously given (7).

A solution of 0.2 gm. of gitoxigenin in a mixture of 4 cc. of acetic acid and 1 cc. of water was treated with 1 cc. of 20 per cent aqueous $CrO_3$, a decided excess of reagent. After 15 minutes at 25° the diluted mixture was extracted with chloroform. The latter after washing with water and finally with dilute $Na_2CO_3$ was dried and concentrated. The residue crystallized under acetone and was collected with a few drops of acetone followed by acetone-ether (1:1). For recrystallization the concentrated acetone solution was carefully treated with dry ether. The α compound formed rhombic plates and prisms which melted at 200° after preliminary softening. When recrystallized from acetone alone, in which the substance is easily soluble, the melting point was raised to 204°. Contrary to gitoxigenin the dioxo derivative no longer gives the nitroprusside reaction.

$[\alpha]_D^{25} = +88$ (c = 0.386 in acetone).

4.460 mg. substance: 3.095 mg. $H_2O$, 11.660 mg. $CO_2$.

$C_{23}H_{30}O_5$. Calculated. C 71.46, H 7.83.

Found. " 71.30, " 7.77.

An appreciable amount of acid material which resulted during the oxidation was recovered from the carbonate extract but proved to be amorphous and resisted successful inquiry.

The α compound is isomerized to the β compound on treatment with dilute mineral acid or on saponification with NaOH and subsequent reacidification with acetic acid.

If, as originally published, the $CrO_3$ solution contains $H_2SO_4$ the substance resulting from the oxidation of gitoxigenin proved to be principally the β compound with smaller amounts of the α-isomer. The amount of the latter increased if the oxidation was carried on at low temperature and for a shorter time.

In one experiment 4 gm. of gitoxigenin were dissolved in 80 cc. of acetic acid and 20 cc. of water. The solution was treated with 20 cc. of Kiliani $CrO_3$ solution[1] and was allowed to stand at 5° for 7 minutes. When worked up as given above the product was found to be a mixture. The neutral residue from the chloroform extract was dissolved in acetone and carefully treated with dry ether. The crystalline substance was collected with ether. 2.9 gm. were obtained. When this was recrystallized by concentration of its acetone solution characteristic rhombic platelets of the $\alpha$ compound were obtained which after collection weighed 0.6 gm. This possessed all of the properties of the $\alpha$ derivative.

The material in the mother liquor proved to be a mixture from which on fractional crystallization by careful dilution of the acetone solution the $\beta$-isomer was obtained. This was, however, a tedious process. The $\beta$-isomer greatly preponderated and was more readily obtained if the oxidation was carried on at higher temperature and for a longer time as follows:

*$\beta$-Dioxo Derivative* (*$\beta$-(Oxoisodigitoxigenon)*).—The above reactants were kept at 27° for 1 hour and the resulting mixture was then worked up as usual. Careful addition of dry ether to the concentrated chloroform solution caused the gradual separation of needles which were collected with ether. After recrystallization from chloroform-ether the substance contained ether of crystallization and melted and slowly frothed up at 127–130°.

From dilute acetone the substance was obtained in anhydrous form and melted with effervescence at 213°.

$$[\alpha]_D^{20} = +154 \text{ (c = 0.676 in acetone).}$$

For analysis the substance from chloroform-ether was dried first at 85° and finally at 105° and 15 mm.

4.365 mg. substance: 3.175 mg. $H_2O$, 11.400 mg. $CO_2$.

$C_{23}H_{30}O_5$. Calculated. C 71.46, H 7.83.

Found. " 71.16, " 8.13.

Like the $\alpha$ compound the $\beta$-isomer does not give the nitroprusside reaction. The failure to obtain an anhydro derivative by the action of acid under conditions which at once transformed gitoxi-

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[1] The solution consisted of 400 gm. of $H_2O$, 80 gm. of $H_2SO_4$, and 53 gm. of $CrO_3$.

genin into the dianhydro derivative supports the interpretation that these oxidation products of gitoxigenin are isogitoxigenin derivatives in which the tertiary hydroxyl is bound in oxidic form. When the β compound was dissolved in HCl (1.19) and kept at 20° for 15 minutes the resulting solution on dilution gave an amorphous precipitate which could not be made to crystallize and which represented deep seated alterations. But from the clear filtrate a relatively small amount of crystalline material slowly deposited. After collection with water it was recrystallized by careful dilution of the acetone solution. It melted at 207–208° and proved to be starting material.

$[\alpha]_D^{20} = +166$ (c = 0.217 in acetone).

4.020 mg. substance: 2.850 mg. $H_2O$, 10.477 mg. $CO_2$.
Found. C 71 08, H 7.93.

The β compound was also obtained directly from the α form by the action of dilute acid as follows: 0.1 gm. of α-(oxoisodigitoxigenon) was dissolved in a mixture of 2 cc. of acetic acid and 0.5 cc. of $H_2O$. The solution was then treated with 0.5 cc. of 25 per cent $H_2SO_4$. The change in rotation of the mixture kept at 20° was followed. From an initial direct reading in a 1 dm. tube of $\alpha = +2.18$, after 17 hours the reading had increased to +5.03. The reaction was then discontinued and the substance was extracted from the diluted mixture with chloroform. After recrystallization from dilute acetone it formed prisms which melted with effervescence at 213°.

$[\alpha]_D^{20} = +170$ (c = 0.507 in acetone).

4.600 mg. substance: 3.210 mg. $H_2O$, 12.050 mg. $CO_2$.
Found. C 71.45, H 7.81.

The α form was converted into the β-isomer by the action of alkali as follows: 0.1 gm. of the α compound dissolved in 2 cc. of acetone was treated with 2 cc. of 2 per cent NaOH. After solution had occurred the mixture was allowed to stand for 2 hours at 25°. After dilution and reacidification with acetic acid it slowly crystallized. The substance was collected with water and because of the small amount it was analyzed directly. It melted at 206°

and proved to be the $\beta$ form since $[\alpha]_D^{20} = +166$ (c = 0.350 in acetone).

4.533 mg. substance: 3.210 mg. $H_2O$, 11.750 mg. $CO_2$.
Found. C 70.70, H 7.93.

*Dimethyl Acetal of $\beta$-(Oxoisodigitoxigenon).*—When the attempt was made to recrystallize either the $\alpha$ or $\beta$ compounds from methyl alcohol, the resulting substance was found to contain methoxyl. The addition of methyl alcohol was, however, incomplete and a mixture resulted which melted higher than the starting material. The acetal was prepared as follows: 0.1 gm. of the $\beta$ form was heated in 10 cc. of anhydrous methyl alcohol in a sealed tube at 100° for 18 hours. On rubbing, a copious crystallization gradually separated. After recrystallization from methyl alcohol in which the methylal is sparingly soluble, it formed flat needles and platelets which melted with effervescence at 226–227°.

4.392 mg. substance: 3.240 mg. $H_2O$, 11.200 mg. $CO_2$.
3.595 " " : 3.855 " AgI.
$C_{25}H_{36}O_6$. Calculated. C 69.40, H 8.39, $OCH_3$ 14.34.
Found. " 69.55, " 8.25, " 14.15.

The above reaction was not complete and the material contained in the mother liquors was found to be low in methoxyl content.

After saponification of the acetal and careful reacidification with dilute acetic acid, a neutral substance was recovered which proved to be starting material. If the action of acid is prolonged, however, hydrolysis of the acetal group occurs with formation of the free ketone, as shown in the following experiment.

A solution of 0.1 gm. of the acetal in 20 cc. of warm acetone was treated with 20 cc. of water containing 1 cc. of acetic acid. The clear solution was left at room temperature for a day. On concentration the substance crystallized. After recrystallization from dilute acetone it melted with effervescence at 213° and proved to be methoxyl-free.

*Dimethyl Acetal of Isogitoxigenon.*—Isogitoxigenon (1) was heated with 50 parts of dry methyl alcohol at 100° for 20 hours. On concentration to small volume the mixture crystallized slowly and incompletely at 0°. As it separates from methyl alcohol the substance melts at 217° with effervescence. On recrystallization from acetone it forms leaflets which melt at 235°. Contrary to the acetal of β-(oxoisodigitoxigenon) this substance separated in almost pure form from the concentrated reaction mixture, leaving in solution a large proportion of more soluble partially methylated material.

4.208 mg. substance: 3.277 mg. $H_2O$, 10.655 mg. $CO_2$.
$C_{25}H_{38}O_6$. Calculated. C 69.08, H 8.82, $OCH_3$ 14.27.
Found. " 69.06, " 8.71, " 13.58.

The acetal nature of this substance was shown by its instability towards acetic acid. 0.04 gm. was dissolved in 5 cc. of acetone and then treated with 5 cc. of 10 per cent acetic acid. The crystals of acetal which reprecipitated soon redissolved due to hydrolysis. On concentrating to remove acetone, a substance crystallized which melted at 280° and was methoxyl-free. It was, therefore, regenerated isogitoxigenon.

### *Oxidation of Dihydrogitoxigenin.*

*α-Dioxodihydro Derivative (α-(Oxodihydrodigitoxigenon)).*—2.8 gm. of dihydrogitoxigenin were dissolved in a mixture of 60 cc. of acetic acid and 15 cc. of water and then treated with 15 cc. of 20 per cent aqueous $CrO_3$. Oxidation occurred fairly promptly and after 40 minutes at 25–30° the mixture was diluted and extracted with chloroform. The chloroform extract was repeatedly washed with water to remove acetic acid and then finally with water to which just sufficient $Na_2CO_3$ was added to make it faintly alkaline to phenolphthalein. This process extracted the acid oxidation product which will be described below.

The dried chloroform solution of the neutral fraction on concentration left a resin which crystallized when treated with a small amount of acetone in which it is appreciably soluble. After collection with a small amount of chilled acetone it was recrystallized by careful addition of dry ether to the concentrated acetone solution. It formed platelets and prisms which softened to a paste at 200° and melted at 220–221°.

$[\alpha]_D^{20} = +89$ (c = 0.347 in acetone).

3.420 mg. substance: 2.550 mg. $H_2O$, 8.885 mg. $CO_2$.
$C_{23}H_{32}O_5$. Calculated. C 71.08, H 8.31.
Found. " 70.86, " 8.34.

This dioxo compound is isomerized with simultaneous loss of water under the influence of mineral acid or alkali to form the following anhydro derivatives.

*Anhydro-β-(Oxodihydrodigitoxigenon).*—0.08 gm. of the above dihydrodioxo compound was dissolved in 2 cc. of acetic acid and 1 cc. of 10 per cent HCl. The rotation was then followed in a 1 dm. tube and was found to increase from an initial $\alpha = +2.00$ to $+3.31$ after 22 hours. At this point it was diluted and extracted with chloroform. The washed chloroform extract left, on concentration, a resin which readily crystallized under acetone. On recrystallization from acetone in which it is quite soluble, it formed platelets and needles which melted at 240°. It was obtained in the same form also from acetone-ether.

$[\alpha]_D^{20} = +154$ (c = 0.28 in acetone).

4.165 mg. substance: 3.020 mg. $H_2O$, 11.380 mg. $CO_2$.
$C_{23}H_{30}O_4$. Calculated. C 74.54, H 8.17.
Found. " 74.52, " 8.11.

This substance was saponified by solution in a mixture of equal parts of 4 per cent NaOH and acetone and the solution was allowed to stand for several hours. After acidification the relactonized substance crystallized. On recrystallization from acetone by addition of ether it formed needles which melted at 219°. This melting point remained the same after several recrystallizations. This suggested that a further isomerization of the anhydro compound in addition to the α to β transformation occurs under these conditions.

The same isomeric substance was also obtained by the action of alkali directly on α-(oxodihydrodigitoxigenon) as follows: 0.1 gm. of the α-dihydrodioxo compound was treated with a mixture of 2 cc. of 2 per cent NaOH and 1 cc. of alcohol. Solution gradually occurred due to saponification of the lactone. After 1 hour at 25–30° the mixture was diluted and acidified gently with acetic acid. As relactonization occurred, the substance gradually crystallized. On recrystallization from acetone-ether it formed needles which melted at 220°.

$[\alpha]_D^{27} = +173$ (c = 0.359 in acetone).

3.957 mg. substance: 2.840 mg. $H_2O$, 10.772 mg. $CO_2$.
$C_{23}H_{30}O_4$. Calculated. C 74.54, H 8.17.
Found. " 74.25, " 8.03.

*Acid*, $C_{23}H_{32}O_6$.—The dilute $Na_2CO_3$ extract of the acid material obtained from the above oxidation mixture was concentrated under diminished pressure to a small volume. On gentle acidification with acetic acid a gum was precipitated. On being rubbed and left to stand this was followed by crystallization of micro rhombs and prisms with gradual crystallization of the gum. After 24 hours this was collected. The yield was 0.6 gm. An additional 0.3 gm. was obtained by the addition of saturated ammonium sulfate to the mother liquor. The acid was dissolved by the addition of a few drops of water to a suspension in warm acetone. After concentration it crystallized as compact prisms which melted at 246° with effervescence. The acid is sparingly soluble in dry acetone and chloroform and readily soluble in alcohol.

$[\alpha]_D^{25} = -74$ (c = 0.377 in acetone-water (4:1)).

For analysis and titration the substance was dried at 100° and 15 mm.

Sample a. 4.265 mg. substance: 3.053 mg. $H_2O$, 10.645 mg. $CO_2$.
" b. 4.374 " " : 3.105 " " 10.965 " "
$C_{23}H_{32}O_6$. Calculated. C 68.27, H 7.98.
Found. Sample a. " 68.07, " 8.01.
" b. " 68.35, " 7.94.

10.830 mg. of substance were treated with 1 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found, 0.261 cc. Calculated for 1 equivalent, 0.268 cc.

12.747 mg. of substance on direct titration required 0.317 cc. Calculated for 1 equivalent, 0.315 cc. 3.0 cc. of 0.1 N NaOH were then added and after being refluxed for 2 hours the mixture was titrated back. Found, 0.052 cc. or only 16.5 per cent of the theory for an extra equivalent.

92.480 mg. of substance were heated in a sealed tube with 1 cc. of alcohol and 4.877 cc. of N NaOH at 130° for 5½ hours and then titrated back. Found, 0.251 cc. Calculated for 1 equivalent, 0.229 cc. The presence of a lactone group, therefore, could not be directly determined.

In working up the acid fraction of this substance, dilute $Na_2CO_3$, which was definitely alkaline to phenolphthalein, was used and therefore a possibility appeared that saponification first occurred and then relactonization after acidification on another carboxyl. In order to obviate such a possibility dilute $NaHCO_3$ was used to extract the acid, the solution never reaching an alkalinity sufficient to turn phenolphthalein. The acid obtained in this experiment proved to be identical in all respects with the above acid.

In an attempt to detect the possible retention of the tertiary hydroxyl group in this acid, its behavior towards HCl was examined as follows: 0.1 gm. of the acid was dissolved in 2 cc. of HCl (1.19) at 0°. After 15 minutes the solution was diluted, which caused the separation of some amorphous material. The filtrate on treatment with ammonium sulfate solution deposited a crystalline substance which was recrystallized from dilute acetone. It melted at 247° and proved to be starting material.

$[\alpha]_D^{25} = -75$ (c = 0.36 in acetone-water (4:1)).

4.360 mg. substance: 3.250 mg. $H_2O$, 10.870 mg. $CO_2$.
Found. C 68.00, H 8.34.

With the idea that the neutral α-dioxo compound might be intermediate in the formation of this acid the attempt was made to reoxidize the former with $CrO_3$. No acid product was obtained and α-(oxodihydrodigitoxigenon) was recovered.

*Methyl Ester of the Acid,* $C_{23}H_{32}O_6$.—This was prepared from the acid with diazomethane in acetone solution. On recrystallization from dilute methyl alcohol it formed platelets which melted at 212°.

4.337 mg. substance: 3.230 mg. $H_2O$, 10.960 mg. $CO_2$.
3.772 " " : 2.140 " AgI.
$C_{24}H_{34}O_6$. Calculated. C 68.86, H 8.19, $OCH_3$ 7.41.
Found. " 68.92, " 8.33, " 7.49.

*Isogitoxigenin Benzoate.*—Isogitoxigenin was benzoylated in pyridine solution with an excess of benzoyl chloride. The mixture was allowed to stand about 18 hours and then poured into dilute $H_2SO_4$. The benzoate readily crystallized from alcohol as needles which melted at 248°.

For analysis the substance was dried at 100° and 15 mm.

5.088 mg. substance: 3.520 mg. $H_2O$, 13.550 mg. $CO_2$.
$C_{30}H_{38}O_6$. Calculated. C 72.83, H 7.75.
Found. " 72.63, " 7.73.

*Gitoxigenin Dibenzoate.*—Gitoxigenin was benzoylated under conditions identical with those used in the case of isogitoxigenin. The substance proved to be a dibenzoate as already reported by Cloetta (4) and Windaus and Schwarte (4).

4.867 mg. substance: 3.125 mg. $H_2O$, 13.188 mg. $CO_2$.
$C_{37}H_{42}O_7$. Calculated. C 74.21, H 7.08.
Found. " 73.91, " 7.18.

*Dihydrodigitoxigenon.*—Dihydrodigitoxigenin was oxidized in 80 per cent acetic acid solution with aqueous $CrO_3$. After 15 minutes at 8° the diluted mixture was extracted with chloroform. After the extract was washed with water and dilute carbonate the ketone was readily obtained on concentration. It formed needles from dilute acetone which melted at 160–162°.

4.605 mg. substance: 3.750 mg. $H_2O$, 12.505 mg. $CO_2$.
$C_{23}H_{34}O_4$. Calculated. C 73.75, H 9.15.
Found. " 74.06, " 9.11.

The acid fraction which was extracted with dilute carbonate was negligible in amount.

The ketone was gently saponified by solution in 2 per cent NaOH to which a half volume of alcohol was added. After dilution and gentle acidification with acetic acid it slowly crystallized. This effervesced at 163° and proved to be starting material and not an anhydro derivative as in the case of the corresponding dihydrogitoxigenin derivative.

5.260 mg. substance: 4.420 mg. $H_2O$, 14.300 mg. $CO_2$.
Found. C 74.14, H 9.39.

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# TIGOGENIN, A DIGITALIS SAPOGENIN.

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Digitonin and in smaller amount gitonin are the principal saponins of digitalis seeds. In recent work Windaus (1) and coworkers have made observations which indicate that still other saponins must be present. Although the latter were not isolated as such their presence was detected by the isolation of characteristic degradation products. When the pure sapogenins, digitogenin or gitogenin, were oxidized with chromic acid only acid substances, digitogenic acid and gitogenic acid were obtained respectively, with no neutral by-products. If, however, a digitogenin was used which had been obtained from crude digitonin, then an appreciable amount of neutral fraction was secured. Most of this consisted of a diketone, $C_{26}H_{38}O_4$, which was presumably an oxidation product of a dihydroxy compound, $C_{26}H_{42}O_4$, an isomer of gitogenin and in which the hydroxyl groups are not vicinal as in gitogenin. In still much smaller amount a neutral monoketone was obtained which was apparently an oxidation product of the secondary alcoholic group of still another sapogenin, $C_{26}H_{42}O_3$, and therefore isomeric with sarsapogenin. The sapogenins themselves were not isolated.

These conclusions have been substantiated in the case of the sapogenin, $C_{26}H_{42}O_3$, by the following observations which we have been able to make. In connection with one of our problems we have had occasion to prepare gitogenin by the hydrolysis of the saponin which we had collected in the course of the preparation of the cardiac glucosides from the leaves of *Digitalis purpurea*. Crude gitonin was found to crystallize in characteristic leaflets on concentration of the purified 50 per cent alcoholic extract of the leaves. When the crude saponin was hydrolyzed, the resulting sapogenin was found to melt too low for gitogenin and the analytical figures were consistently high in carbon. It was then found possible to separate the contaminant from gitogenin by taking advantage of the greater solubility of the former in petrolic ether. By this means gitogenin was readily obtained in pure form. The more soluble substance was also readily purified and on analysis proved to possess the formula $C_{26}H_{42}O_3$. This substance, which may be conveniently called *tigogenin*, proved to be a secondary alcohol yielding on oxidation a ketone which is probably identical with the ketone, $C_{26}H_{40}O_3$, of Windaus and Willerding. *Tigogenone* yielded a monooxime. Tigogenin gave a *monoacetate* and a *monobenzoate*. As in the case of digitogenin and gitogenin, the remaining two oxygens cannot be characterized directly and appear to be of oxidic character.

A comparison of sarsapogenin from the saponin of sarsaparilla root as well as its ketone sarsapogenone with tigogenin and its derivatives has shown that these sapogenins are isomeric. The substances which resulted by the reduction of the ketones according to the method of Clemmensen also proved to be different.

## EXPERIMENTAL.

*Tigogenin.*—2 kilos of *Digitalis purpurea* leaves, after preliminary extraction with water, were extracted twice with 6 liters of 50 per cent alcohol. The alcoholic extracts were precipitated with basic lead acetate, and the filtrate was freed from the excess of lead by careful addition of ammonium sulfate. The filtrate from $PbSO_4$ was concentrated under diminished pressure to remove the alcohol. The shining platelets which separated were collected with water. This product, which contained considerable colored impurity, was dissolved in a mixture of 500 cc. of 50 per cent alcohol and 50 cc. of HCl (1.19). The solution was refluxed for 3 hours and on cooling a dark green product was obtained. This was collected with water and then dissolved in 100 cc. of alcohol and 15 cc. of concentrated HCl and the solution was again heated for 3 hours. The crystals which separated on dilution with water were repeatedly recrystallized from a small volume of alcohol until the product was freed from colored impurities. This procedure yielded 1.3 gm. of a mixture of tigogenin and gitogenin which melted between 240 and 250°. An additional amount of this mixture of genins was obtained by extraction of the lead sulfate precipitate with 50 per cent alcohol and working up this extract in a manner similar to that outlined above.

A chloroform solution saturated with this mixture at room temperature was precipitated by the addition of 2 volumes of petrol ether (b.p. 40–60°). This precipitate consisted of gitogenin which was still slightly contaminated since it melted at 260–262°.

4.800 mg. substance: 4.280 mg. $H_2O$, 13.100 mg. $CO_2$.
$C_{26}H_{42}O_4$. Calculated. C 74.58, H 10.12.
Found. " 74.43, " 9.98.

The mother liquor was evaporated to dryness and the process was repeated until no more gitogenin could be obtained. The final residue was recrystallized several times from acetone from which it separated in the form of prisms which melted at 203–204°. About 30 to 40 per cent of the mixture of genins was found to be tigogenin.

Tigogenin is soluble in all of the ordinary solvents. It proved to be more soluble in acetone, ether, ligroin (b. p. 80–90°), and petrol ether (b.p. 40–60°) than is gitogenin. This difference in solubility is most marked in the case of petrol ether (b.p. 40–60°).

The cholesterol test gave a light yellow color which turned to a reddish brown when the solution was warmed.

$[\alpha]_D^{25} = -49$ (c = 1.023 in pyridine).

For analysis the substance was dried at 100° and 30 mm.

4.856 mg. substance: 4.632 mg. $H_2O$, 13.847 mg. $CO_2$.
4.907 " " : 4.705 " " 13.950 " "
$C_{26}H_{42}O_3$. Calculated. C 77.55, H 10.52.
Found. (*a*) " 77.77, " 10.67.
(*b*) " 77.53, " 10.72.

The molecular weight determination was made according to the method of Rast.

30.547 mg. camphor: 3.118 mg. anhydrous substance, $\Delta = 11.75$. Mol. wt. calculated, 402.3; found, 382.

*Tigogenin Acetate.*—A solution of 0.2 gm. of tigogenin and an equal amount of fused sodium acetate in acetic anhydride was refluxed for 5 hours. After dilution with water the acetate separated in the form of shining plates. After recrystallization from alcohol it melted at 200–202°.

$[\alpha]_D^{24} = -57$ (c = 1.023 in pyridine).

4.365 mg. substance: 3.912 mg. $H_2O$, 12.110 mg. $CO_2$.
4.880 " " : 4.450 " " 13.570 " "
$C_{28}H_{44}O_4$. Calculated. C 75.62, H 9.97.
Found. (*a*) " 75.67, " 10.03.
(*b*) " 75.83, " 10.20.

*Tigogenin Benzoate.*—A benzene solution of 0.1 gm. of tigogenin was refluxed for 2 hours with an excess of benzoyl chloride and pyridine. After concentrating the washed benzene solution, the residue was recrystallized from acetone. The benzoate separated in the form of plates which melted at 224–225°.

$[\alpha]_D^{26} = -37$ (c = 1.027 in pyridine).

3.965 mg. substance: 3.360 mg. $H_2O$, 11.410 mg. $CO_2$.
3.367 " " : 2.860 " " 9.690 " "
$C_{33}H_{46}O_4$. Calculated. C 78.21, H 9.16.
Found. (*a*) " 78.48, " 9.48.
(*b*) " 78.49, " 9.53.

*Tigogenone.*—An acetic acid solution of 0.1 gm. of $CrO_3$ was added to a solution of 0.3 gm. of tigogenin in 10 cc. of acetic acid and the reaction mixture was heated on a steam bath for ½ hour. The solution was then diluted with 2 volumes of water and the product extracted with ether. The washed ether solution was evaporated to dryness and the residue was recrystallized from acetone. The substance separated in the form of plates which melted at 206–207° (204–205° according to Windaus (1)).

$[\alpha]_D^{24} = -35$ (c = 1.000 in pyridine).

4.810 mg. substance: 4.315 mg. $H_2O$, 13.785 mg. $CO_2$.
5.447 " " : 4.960 " " 15.620 " "
$C_{26}H_{40}O_3$. Calculated. C 77.94, H 10.07.
Found. (*a*) " 78.15, " 10.04.
(*b*) " 78.22, " 10.19.

*Tigogenone Oxime.*—A solution of 0.1 gm. of tigogenone in absolute alcohol was refluxed with an excess of hydroxylamine hydrochloride and sodium acetate for 3 hours. The alcohol was then removed and the residue was recrystallized from acetone. The oxime separated in the form of needles which melted at 256–258° with decomposition.

3.973 mg. substance: 3.595 mg. $H_2O$, 10.992 mg. $CO_2$.
4.212 " " : 3.855 " " 11.655 " "
5.000 " " : 0.142 cc. N (28°, 759 mm.)
$C_{26}H_{41}O_3N$. Calculated. C 75.13, H 9.95, N 3.37.
Found. (*a*) " 75.46, " 10.12.
(*b*) " 75.47, " 10.24.
(*c*) N 3.22.

*Reduction of Tigogenone by Clemmensen's Method.*—A solution of 0.1 gm. of tigogenone in 10 cc. of acetic acid was refluxed with 4 gm. of amalgamated zinc and 2 cc. of HCl (1.19) for ½ hour. The solution was diluted with 3 volumes of water and extracted with ether. The residue from this extract was crystallized from acetone. The product was recrystallized from methyl alcohol from which it separated as leaflets which melted at 265–267°. The yield was very poor.

4.050 mg. substance: 4.095 mg. $H_2O$, 12.065 mg. $CO_2$.
1.283 " " : 1.252 " " 3.780 " "
$C_{26}H_{42}O_2$. Calculated. C 80.76, H 10.96.
Found. (*a*) " 81.24, " 11.30.
(*b*) " 80.35, " 10.92.

*Sarsapogenone.*—0.3 gm. of sarsapogenin, which had been prepared (2) from commercial Honduras sarsaparilla root, was dissolved in 10 cc. of acetic acid. This was oxidized according to the procedure used in the case of tigogenin. The product was finally recrystallized from acetone from which it separated in the form of shining plates which melted at 220–222°.

$[\alpha]_D^{25} = -46$ (c = 1.027 in pyridine).

4.334 mg. substance: 4.020 mg. $H_2O$, 12.438 mg. $CO_2$.
3.956 " " : 3.670 " " 11.345 " "
$C_{26}H_{40}O_3$. Calculated. C 77.94, H 10.07.
Found. (*a*) " 78.27, " 10.38.
(*b*) " 78.20, " 10.37.

*Sarsapogenone Oxime.*—The oxime was prepared as in the case of tigogenone. The product was recrystallized from acetone from which it separated in the form of plates which melted at 126–128°.

3.967 mg. substance: 3.590 mg. $H_2O$, 10.992 mg. $CO_2$.
4.716 " " : 4.260 " " 13.065 " "
5.410 " " : 0.155 cc. N (27°, 759.5 mm.).
$C_{26}H_{41}O_3N$. Calculated. C 75.13, H 9.95, N 3.37.
Found. (*a*) " 75.55, " 10.12.
(*b*) " 75.56, " 10.11.
(*c*) N 3.26.

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# THE APPLICATION OF THE QUINHYDRONE ELECTRODE TO THE DETERMINATION OF THE pH OF SERUM AND PLASMA.*

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## INTRODUCTION.

The need for an accurate method for determining the pH of small amounts of biological fluids with a minimum expenditure of time has resulted in considerable search for a substitute for the hydrogen electrode. Colorimetric methods for determining the pH of serum and plasma were especially devised to meet this need (1, 2). But several investigators (3–5) have been unable to obtain reliable results with the colorimetric procedure, and the methods have come to be regarded as insufficiently accurate for careful experimental work. The quinhydrone electrode seemed worthy of consideration. Although its use in whole blood is still a mooted question, most investigators agree that it can be used successfully with human serum and plasma. In view of the fact that the dog is so widely used as an experimental animal, we were prompted to make a critical study of the applicability of this method to dog serum. 38° was selected as the working temperature since all conversions of the pH of serum from 20–38° are subject to a temperature correction which on theoretical grounds alone should not be regarded as constant under a variety of experimental conditions.

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## *Theoretical.*

The theory of oxidation-reduction potentials and their relation to pH has been discussed many times by different writers. The reader is therefore referred to the excellent reviews on the subject by Clark (6, 7) and by Biilmann (8). In acid or weakly alkaline solutions, the simplified equation for the quinhydrone electrode is as follows:

$$E_h = E_Q + \frac{RT}{F} \ln [H^+] \qquad (1)$$

where $E_Q$ is the characteristic constant of the quinhydrone system expressed in volts. At 20° the value is 0.7029 and at 38° 0.6896 (Clark (6)). We were able to duplicate these values repeatedly in standardizations of the electrode by means of buffer solutions.

Blood serum is just alkaline enough to be slightly beyond the range where the simplified equation holds, but it is possible to apply a correction factor, $E_c$. The modified equation becomes:

$$E_h = E_Q + E_c + \frac{RT}{F} \ln [H^+] \qquad (2)$$

Thus, while $E_c$ remains zero in acid solution, in going up the pH scale beyond 7.0 it assumes a gradually increasing positive value. The point on the pH scale where this occurs is shifted by the temperature at which the electrode is used. For example, at 20° in a 0.2 M phosphate buffer solution, the point where $E_c$ begins to be appreciable in value is about pH 7.6; at 38° the point is about pH 7.0. It becomes at once apparent that at 20° it is possible to determine the pH of most biological fluids with the quinhydrone electrode on the assumption that $E_c$ remains zero (9, 10). At 38°, however, this assumption cannot be made. Since $E_c$ is a function of the pH it was determined experimentally at 20° and 38° in the range 7.0 to 8.0 with a series of phosphate buffers of known pH. The results of these standardizations are the two curves shown in Fig. 1 (*cf.* La Mer and Parsons (11)).

*Calculation of pH from Quinhydrone Electrode Readings.*—Routine calculations are made by the use of the following equation derived from Equation 2.

$$\text{pH} = \frac{E_Q - (\text{E.M.F.} + e_{\text{sat. cal.}})}{0.0001983\ T} + \frac{E_c}{0.0001983\ T} \qquad (3)$$

where E.M.F. represents the observed voltage, and (E.M.F. + $e_{\text{sat. cal.}}$) = $E_h$. The above equation may serve at any temperature, provided the proper values of $E_Q$ and $e_{\text{sat. cal.}}$ are used. If the pH is to be determined within the range where $E_c$ is appreciable, it has been customary to proceed as follows: (1) Calculate the approximate pH of the solution with the above equation, $E_c$ being assumed to be zero. (2) From one of the curves in Fig. 1 read off $E_c$ corresponding to this pH. (3) Add to the approximate pH this value of $E_c$ (expressed in pH units). The pH thus determined is assumed to represent the pH of the solution.

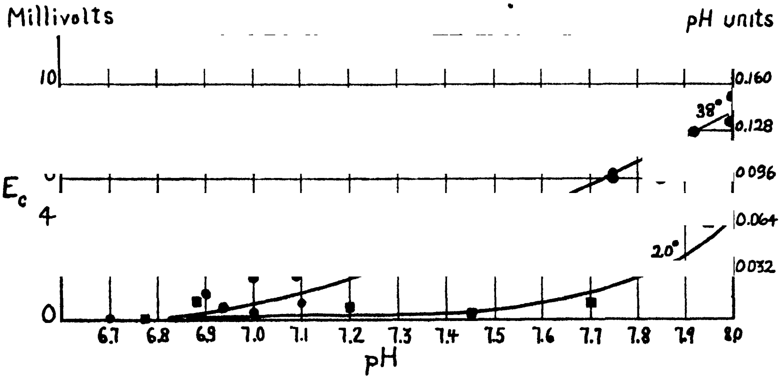

FIG. 1. The variation of $E_c$ with pH at 20° and 38°. pH units (ordinate) = $\frac{E_c}{0.000198T}$

## Apparatus.

The hydrogen electrode apparatus and accessory equipment were standard. The chambers were the Clark-Cullen 2 cc. rocking type. The saturated calomel half-cell was the standard reference cell. Duplicate set-ups were available for simultaneous determinations at 20° and 38°.

For the quinhydrone electrode, special micro capillary chambers of Pyrex glass, having a capacity of about 0.1 cc., were devised. These chambers consisted of a barrel large enough to admit the glass tube carrying the electrode, and an accurately centered capillary 25 mm. long and 1.5 mm. in diameter. The end of the capillary was constricted somewhat to diminish diffusion of KCl into the capillary, and to keep solid quinhydrone from dropping out. The electrodes of 1 mm. gold or platinum wire were 20 mm. long and sealed with special glass into glass tubing. The lead was soldered directly to the electrode. No mercury connections were used. When the electrode was put into the chamber, the end was about 10 mm. from the end of the capillary. Fig. 2 shows a sketch

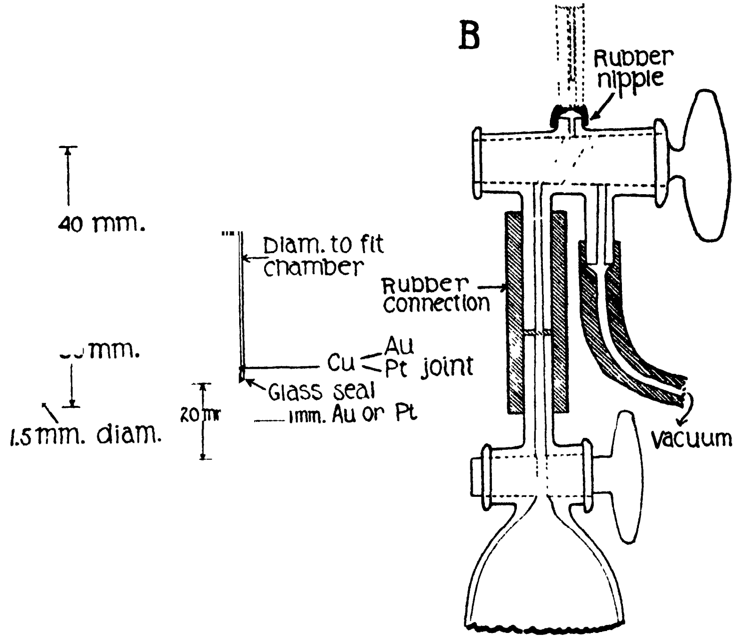

Fig. 2. *A*, details of the quinhydrone electrode and capillary chamber; *B*, filling device.

of the chamber and the electrode. The chambers were filled by forcing the liquid up into the capillary from a tonometer by means of a mercury leveling bulb. The device for accomplishing this without loss of $CO_2$ is shown in Fig. 2. The electrode and vessel are similar in general design to those recently described by Gesell (12) who also makes use of an auxiliary device for forcing the liquid into the chambers. We feel that the use of a plunger type described by Cullen (13) where liquid fills the chamber due to decreased pressure within the chamber may cause loss of $CO_2$. In certain poorly buffered fluids like pancreatic juice, the effect of such loss of $CO_2$ has been shown to occur

### *Methods.*

The hydrogen electrode analyses were standardized with 0.1 N HCl, a pH of 1.076 at 20° and 38° being assumed, and *e* for the entire reference system consisting of the saturated calomel cell plus liquid junction potential was calculated to be 0.2358 volt at 38° and 0.2475 volt at 20°. Hydrogen electrode determinations of serum were made with the Hasselbalch technique.

TABLE I.

*Comparison of pH of Artificial Serum As Determined by Hydrogen and Quinhydrone Electrodes at 38°.*

| | Experiment No. | $pH_{H_2}$ | $pH_Q$ | $pH_{H_2} - pH_Q$ |
|---|---|---|---|---|
| Buffered with phosphate. | 1 | 7.414 | 7.39 | +0.02 |
| | 2 | 7.512 | 7.52 | −0.01 |
| | 3 | 7.380 | 7.35 | +0.03 |
| Unbuffered. | 1 | 7.462 | 7.34 | +0.12 |
| | 2 | 7.574 | 7.50 | +0.07 |
| | 3 | 7.475 | 7.42 | +0.06 |

The electrodes for the quinhydrone determinations were always polished on cork with fine emery before use. After being used on serum, they were put overnight in cleaning solution and then for a day in distilled water. The C.P. platinum electrodes advised by Cullen (13) were found to be no better than those made from ordinary platinum wire. In regard to the use of gas flames in the preparation of the electrodes, we have not detected any poisoning such as might be revealed by erratic behavior.

In making quinhydrone determinations, the following procedure was adopted. Dry quinhydrone was dusted into the capillary chamber with a medicine dropper, care being taken to get approximately the same amount into each. Then the electrode was inserted and connection made to the lead-off wire. In order to prevent waste of material and loss of $CO_2$ in transfer, the stopcock (Fig. 2, *B*) with rubber nipple to accommodate the electrode chamber was fitted to the tonometer containing the serum. After the system was filled with serum and a small amount allowed to run out, the apparatus was ready for use. When a quinhydrone chamber was put in place, the stop-cock was opened slowly and serum allowed to flow into the capillary. The serum was thus forced up beyond the glass seal of the electrode. As soon as the chamber was filled it was suspended in a saturated KCl bridge, so as just to touch the surface of the KCl, and the voltage read on the potentiometer. Excess serum remaining in the nipple and dead space below was sucked off by vacuum on turning the stop-cock through 180°. The electrode was tested for the effect of KCl diffusion and loss of $CO_2$. No noticeable diffusion of KCl into the capillary occurred within 10 minutes, which is ample time for any determination. Loss of $CO_2$ was studied with an "artificial serum" equilibrated at 40 mm. of $CO_2$ tension, consisting of 0.082 M NaCl, 0.022 M $NaHCO_3$, and, where buffer was desired, 0.055 M $NaH_2PO_4$ to give it the buffer capacity of serum. Comparative determinations by the hydrogen and quinhydrone electrodes were made. Table I gives the results of six such experiments. The results with the quinhydrone electrode used on solutions having the buffer capacity of serum checked with those of the hydrogen electrode quite closely. This was not the case, however, with the unbuffered artificial serum.

Fig. 3 shows the behavior of the quinhydrone electrode in 0.2 M phosphate buffers, buffered artificial serum, and unbuffered artificial serum. Although the drift of the electrode in buffered artificial serum is noticeable, and goes in an alkaline direction, which might indicate some loss of $CO_2$, this is nevertheless slight and insignificant when compared with a drift in blood serum due to other causes. But in the unbuffered artificial serum, the electrode exhibits not only an unmistakable drift due apparently to loss of $CO_2$, but also very erratic behavior. This confirms the findings of Cullen and Earle (9) and Kolthoff (14, 15).

For the experiments described below, blood was drawn from dogs by heart puncture and collected under oil. If serum was desired, the blood was allowed to clot in centrifuge tubes; if plasma, enough of a concentrated solution of $K_2C_2O_4$ and NaF 3:1 was added to make a 0.4 per cent solution of the mixed salts. It is a curious fact that hemolysis of dog blood seldom occurs when a concentrated solution of anticoagulant is used, while some hemolysis nearly always is evident when the dry salts are mixed with the blood. The blood was centrifuged under solid paraffin for 20 minutes. The supernatant serum or plasma was drawn off and stored in tonometers over mercury. Transfer was made directly from the tonometers into the hydrogen and quinhydrone electrode chambers.

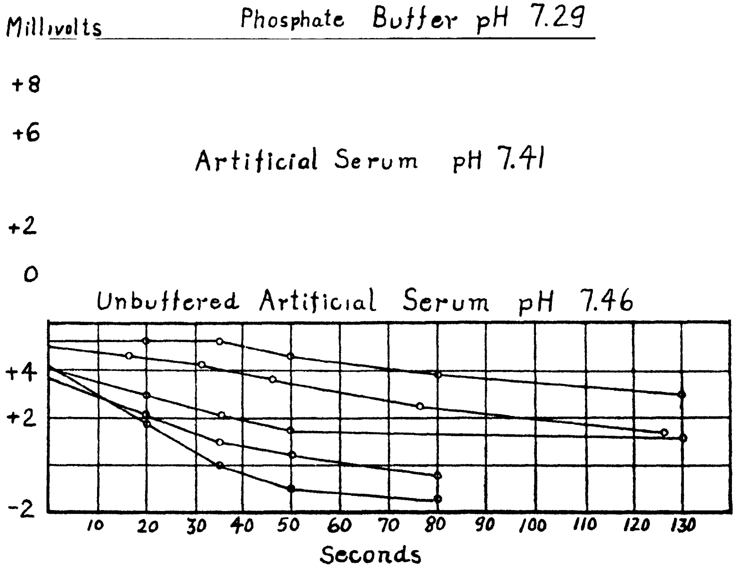

FIG. 3. The drift of the quinhydrone electrode in certain solutions of inorganic salts.

## EXPERIMENTAL.

### *Use of Quinhydrone Electrode on Serum.*

In attempting to determine the pH of serum and plasma with the quinhydrone electrode at 38°, a striking fact presented itself. As soon as the quinhydrone was mixed with serum, a very pronounced drift toward more acid pH occurred which was accompanied by the formation of a pale to deep red color in the capillary. This drift was presumed to be due to a side reaction whereby some constituent of the serum, possibly the protein, combined either with oxidant or reductant. Reimers (16) in some test-tube experiments has shown that a reaction producing a reddish brown precipitate occurs with most protein solutions and quinone, but not

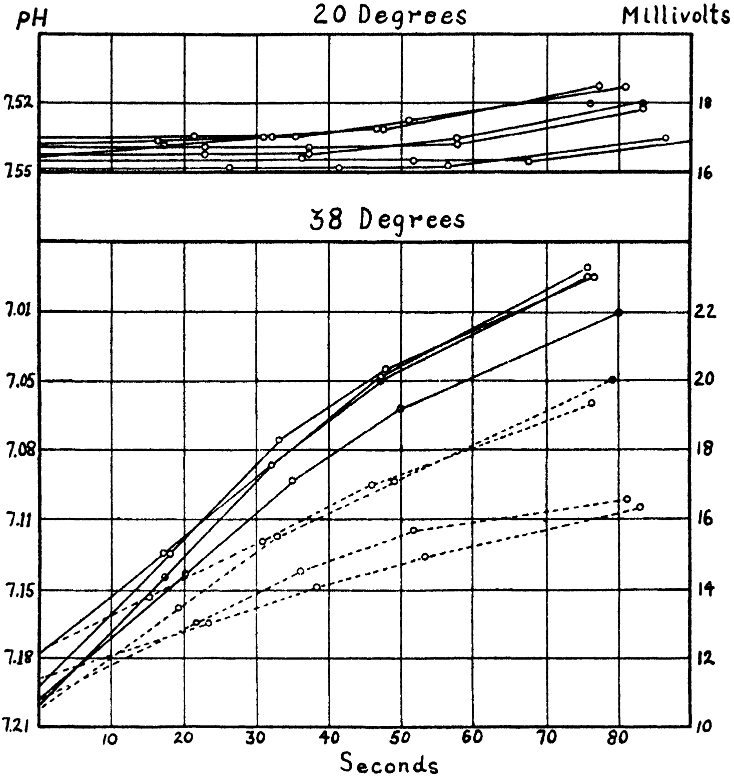

FIG. 4. The drift of the quinhydrone electrode in dog serum at 20° and 38°.

hydroquinone. In addition it was observed that the rate of drift and the degree of color formation were directly proportional to the excess amount of quinhydrone added to the capillary. If the excess quinhydrone over the amount necessary to saturate the serum was great, the drift was increased; if the excess was small, the drift was much reduced. In Fig. 4 are shown the drifts of the quinhydrone electrode in serum at 38° and 20°. The broken lines represent determinations where the excess of quinhydrone was slight, while the solid lines represent cases where larger amounts were used. Whereas the drift at 38° varies from 0.02 to 0.04 pH in 15 seconds, to 0.2 pH in 1 minute, at 20° the observed voltage sometimes remains constant over a period of 30 to 40 seconds before an acid drift begins. It becomes obvious from these results why previous investigators have made observations at room temperature.

Because of the great drift at 38°, no reliable determinations could be made unless the time relations were closely observed. This necessitated special technique. As soon as the serum was forced up into the capillary from the tonometer to mix with the quinhydrone, a stop-watch was started and the electrode suspended in the KCl bridge. Then a reading on the potentiometer was made as rapidly as possible. This interval of time usually averaged 15 seconds, with a single operator. Two more readings were made at 15 seconds intervals, and frequently a third 30 seconds later. By plotting the time against the voltage reading, the lines shown on the graph were obtained. In order to approximate the voltage of the electrode at the moment when contact of the quinhydrone with the serum first occurred, extrapolation to zero time was resorted to. It is to be emphasized that extrapolation to zero along straight lines is probably not entirely correct, but the fact remains that too little is known about the behavior of the electrode in the first few seconds after the quinhydrone has come in contact with the serum to make a mathematical curve extrapolation justifiable. The extrapolated values usually ranged from 0.02 to 0.04 pH more alkaline than the first reading at 15 seconds after mixing, and were assumed to represent the pH of the serum before any complicating side reaction could occur. The lines extrapolated toward zero time always converged. The scattering of the extrapolated values was 0.04 pH. Deviations from the average readings of six or seven different electrodes were rarely greater than 0.02 pH. In fact, a series always grouped itself so that when occasional single erratic results did occur, they could be ignored. It must be emphasized, however, that in a system where such violent drifts do occur, single determinations are of doubtful value. On this account usually from four to six determinations were made and occasionally from ten to twelve. When one is working in this way, each comparison of the pH of serum by the hydrogen and quinhydrone electrode represents two hydrogen electrode values which in 90 per cent of the cases checked each other within 0.01 pH, and generally five or six quinhydrone determinations. The difference between the average values obtained by the two methods was taken to represent the deviation of the quinhydrone electrode reading from the true pH of the serum. Table II represents several typical comparisons. The individual quinhydrone electrodes of gold or platinum are designated by numbers. All pH calculations for the quinhydrone electrode reported in this paper were made from potentials extrapolated to zero time.

TABLE II.

*Typical Experiments Which Illustrate Comparative pH Determinations of Dog Serum at 38° by Hydrogen and Quinhydrone Electrodes.*

| Dog No. | Date. | Hydrogen electrode. | | Quinhydrone electrode. | | | | $pH_{H_2} - pH_Q$ |
|---|---|---|---|---|---|---|---|---|
| | | pH | Average pH. | Electrode No. | pH | | Average pH. | |
| | *1929* | | | | | | | |
| 7 | Aug. 5 | 7.390 | | Au 1 | 7.33 | 7.34 | | |
| | | 7.387 | 7.389 | Au 6 | 7.35 | 7.36 | | |
| | | | | Au 2 | 7.34 | 7.33 | | |
| | | | | Pt 5 | 7.34 | | | |
| | | | | Pt X | 7.34 | | 7.34 | +0.05 |
| 8 | Aug. 6 | 7.321 | | Au 8 | 7.29 | 7.28 | | |
| | | 7.324 | 7.322 | Au 7 | 7.29 | | | |
| | | | | Au 3 | 7.29 | 7.29 | | |
| | | | | Pt 3 | 7.29 | | | |
| | | | | Pt 1 | 7.30 | | 7.29 | +0.03 |
| 2 | July 18 | 7.279 | | Au 6 | 7.27 | 7.27 | | |
| | | 7.279 | 7.279 | Au 5 | 7.28 | 7.29 | | |
| | | | | Au 1 | 7.25 | 7.28 | | |
| | | | | Au 8 | 7.25 | 7.27 | 7.27 | +0.01 |

Table III shows comparative data on the pH of dog serum and plasma of normal dogs by the hydrogen and quinhydrone electrodes. Except in one isolated case, the quinhydrone electrode

TABLE III.

*Comparison of pH of Serum and Plasma of Normal Dogs at 38° by Hydrogen and Quinhydrone Electrodes.*

| Dog No. | Date. | $pH_{H_2}$ | $pH_Q$ | $pH_{H_2} - pH_Q$ | Remarks. |
|---|---|---|---|---|---|
| | *1929* | | | | |
| 2 | July 3 | 7.336 | 7.32 | +0.02* | Animal pregnant. |
| | " 10 | 7.239 | 7.20 | +0.04 | |
| | " 18 | 7.305 | 7.27 | +0.04 | |
| 3 | " 11 | 7.322 | 7.27 | +0.05 | Slight hemolysis. Wk. after injection of NaCN. |
| | Aug. 7 | 7.340 | 7.28 | +0.06 | |
| | " 14 | 7.306 | 7.30 | +0.01 | |
| 4 | July 12 | 7.336 | 7.31 | +0.03 | |
| 6 | " 17 | 7.361 | 7.35 | +0.01 | Slight hemolysis. |
| | " 24 | 7.368 | 7.36 | +0.01 | |
| 7 | Aug. 5 | 7.389 | 7.34 | +0.05 | |
| | " 9 | 7.354 | 7.30 | +0.05 | NaF added to serum. |
| 8 | " 6 | 7.321 | 7.29 | +0.03 | " " " " |
| 11 | Nov. 1 | 7.351 | 7.30 | +0.05 | |
| | " 15 | 7.410 | 7.36 | +0.05 | |
| | *1930* | | | | |
| | Jan. 16 | 7.368 | 7.31 | +0.06 | |
| | Feb. 19 | 7.388 | 7.35 | +0.04 | 48 hrs. after 1 per cent hemorrhage. |
| | Apr. 4 | 7.378 | 7.32 | +0.06 | |
| | *1929* | | | | |
| 12 | Dec. 27 | 7.323 | 7.26 | +0.06 | Blood kept at 38° 35 min. before centrifuging. |
| | " 13 | 7.385 | 7.32 | +0.06 | |
| | *1930* | | | | |
| | Feb. 14 | 7.381 | 7.36 | +0.02 | |
| | " 15 | 7.425 | 7.42 | +0.01 | Mixture of serum and plasma. |
| 15 | Jan. 24 | 7.358 | 7.34 | +0.02 | |
| | Feb. 24 | 7.353 | 7.34 | +0.01 | |
| 16 | " 12 | 7.390 | 7.38 | +0.01 | Mixture of serum and plasma. |
| | " 12 | 7.363 | 7.35 | +0.01 | Plasma. |
| | " 25 | 7.432 | 7.44 | −0.01 | " |
| 17 | Mar. 4 | 7.400 | 7.38 | +0.02 | " on ice overnight. |
| | " 5 | 7.455 | 7.44 | +0.02 | " |
| | Apr. 17 | 7.352 | 7.32 | +0.03 | |
| 18 | Mar. 6 | 7.480 | 7.47 | +0.01 | Plasma. |
| Average | | | | +0.03 | |

* Serum was used unless otherwise specified.

always gave pH values which were more acid than those obtained by the hydrogen electrode. We have chosen to call this difference between the hydrogen and quinhydrone pH values, the *Q* correction throughout the remainder of the paper. The data are arranged to show variation of this correction in individual animals. In thirty-six normal samples from twelve dogs (see also Tables VII and VIII) the *Q* correction averaged +0.03 pH with a maximum spread extending over 0.08 pH. If we assign +0.03 pH as the constant correction of the quinhydrone electrode for normal dogs, then 78 per cent of all the determinations check the hydrogen electrode within ±0.02 pH and 95 per cent within ± 0.03 pH.

### *Effect of Hemorrhage or Injection of Sodium Cyanide.*

In view of the experience in this laboratory with the colorimetric method on dog serum and plasma after hemorrhage (3, 5), some experiments were carried out to see whether hemorrhage or injection of NaCN would cause a variation in the *Q* correction. Gesell and Hertzman (17) in their continuous recording experiments on the pH of dog blood with the manganese dioxide electrode, found after hemorrhage, and especially after injection of 0.01 M NaCN, that the $MnO_2$ electrodes became erratic, but in general, the quinhydrone electrodes agreed with the hydrogen electrode values within 0.05 pH or less. Only occasionally was a larger deviation observed.

If hemorrhage affects the quinhydrone electrode, it may do so in two ways: (1) by lowering the protein concentration through dilution which in turn decreases the buffer capacity of serum; (2) by piling up metabolites resulting from insufficient oxidation. These two effects do not occur simultaneously. The protein concentration does not reach its lowest ebb until 18 to 24 hours after the hemorrhage, when influx of tissue fluids low in proteins has restored the blood volume. Metabolites, on the other hand, pile up rapidly, so that the blood may become more acid than normal within 20 to 30 minutes after hemorrhage. Whether the magnitude of the acid change is a direct measure of the quantity of metabolites present has not been shown, although such a presumption appears probable.

In the five experiments, in Table IV, the effect of hemorrhage on the *Q* correction was studied. In no case was there a change in the

$Q$ correction sufficiently great to be attributed to the immediate effects of the hemorrhage. 24 hours after hemorrhage, when the colorimetric corrections always ran low, it was thought that the $Q$ correction might be high due to diminution of the buffer capacity of the serum. Here again, no effect on the $Q$ correction was noted except in the case of Dog 2.

TABLE IV.

*Effect of Hemorrhage on Quinhydrone Correction.*

| | Date. | $pH_{H_2}$ | $pH_Q$ | $pH_{H_2} - pH_Q$ | Remarks. |
|---|---|---|---|---|---|
| | *1929* | | | | |
| Dog 2, 16.8 kilos. | July 18 | 7 305 | 7.27 | +0.04 | Normal. |
| | | 7.279 | 7.27 | +0.01 | 80 min. after 2.6 per cent hemorrhage.[*] |
| | " 19 | 7.309 | 7.23 | +0.08 | 24 hrs. after 3.1 per cent hemorrhage. |
| Dog 6, 6.7 kilos. | July 24 | 7.368 | 7.36 | +0 01 | Normal. |
| | | 7.380 | 7.35 | +0.03 | 130 min. after 1.9 per cent hemorrhage. |
| | " 25 | 7.390 | 7.38 | +0.01 | 24 hrs. after 3.2 per cent hemorrhage. |
| Dog 7, 18.2 kilos. | Aug. 9 | 7.354 | 7.30 | +0.05 | Normal. |
| | | 7.297 | 7.25 | +0.05 | 20 min. after 3.3 per cent hemorrhage. |
| | " 12 | 7.331 | 7.32 | +0.01 | 3 days after hemorrhage. |
| | *1930* | | | | |
| Dog 15, 19.7 kilos. | Jan. 24 | 7.358 | 7.34 | +0.02 | Normal. |
| | " 25 | 7.438 | 7.41 | +0.04 | 24 hrs. after 3 per cent hemorrhage. |
| | " 29 | 7.382 | 7.36 | +0.02 | 4 days after hemorrhage. |
| Dog 17, 14 kilos. | Apr. 17 | 7.352 | 7.32 | +0.03 | Normal. |
| | | 7.260 | 7.20 | +0.06 | 30 min. after 3.5 per cent hemorrhage. |
| | " 18 | 7.408 | 7.36 | +0.05 | 24 hrs. after. 4 per cent hemorrhage. |

[*] Amounts of blood removed are expressed as per cent of body weight.

The immediate effect of hemorrhage on the occurrence of metabolites in the blood stream may possibly be correlated with the effect of NaCN injection. In this case also, oxidation is interfered with. Usually the blood becomes more acid very soon after the injection of 0.01 N NaCN. Since only two of our experiments listed in Table V show a small acid change, it is not certain to what extent metabolites may have piled up. On the other hand, since we observed more or less severe hyperpnea in all dogs injected, and regarded it as a satisfactory indication of the NaCN effect, this may have somewhat masked the acid swing. In two experiments, the injection of NaCN did not affect the *Q* correction. In the last experiment (Dog 3), however, a change in the *Q* correction appeared. The fact that in this determination the hydrogen electrode gave somewhat erratic results does not allow us to place too much weight on the experiment.

TABLE V.

*Effect of Injection of Sodium Cyanide on Quinhydrone Correction.*

| | Date. | $pH_{H_2}$ | $pH_Q$ | $pH_{H_2} - pH_Q$ | Remarks. |
|---|---|---|---|---|---|
| | *1929* | | | | |
| Dog 7, 18.2 kilos. | Aug. 5 | 7.389 | 7.34 | +0.05 | Normal. |
| | | 7.343 | 7.33 | +0.01 | 5½ min. after injection of 18 cc. 0.01 M NaCN. |
| Dog 8, 17.7 kilos. | Aug. 6 | 7.321 | 7.29 | +0.03 | Normal. |
| | | 7.331 | 7.29 | +0.04 | 72 sec. after injection of 15 cc. 0.01 M NaCN. |
| Dog 3, 11.6 kilos. | Aug. 7 | 7.340 | 7.28 | +0.06 | Normal. |
| | | 7.31* | 7.19 | +0.12 | 45 sec. after injection of 14.5 cc. 0.01 M NaCN. Convulsions. |

* Single determination.

## *Effect of Allowing Blood to Stand before Centrifuging.*

All sera for pH determinations were obtained from blood which had been allowed to clot at room temperature for about 10 minutes before centrifuging. It seemed possible that serum separated after longer contact with the corpuscles, especially if the blood was kept at high temperatures, might contain certain substances which would exert a disturbing influence on the quinhydrone electrode. To test this possibility, one sample of blood was drawn into cold centrifuge tubes, allowed to clot 10 minutes at room temperature, and the serum separated as rapidly as possible. A second sample was allowed to stand varying lengths of time both at room temperature and at 28–38°. The results of these experiments are given in Table VI. In four cases the quinhydrone correction rose. The incidence of glycolysis can be plainly seen upon comparing the pH of the sera obtained by the routine method with the

TABLE VI.

*Increase in Quinhydrone Correction of Serum When Blood Is Allowed to Stand at High Temperatures before Centrifuging.*

| Dog No. | Date. | $pH_{H_2}$ | $pH_Q$ | $pH_{H_2} - pH_Q$ | Treatment of blood. |
|---|---|---|---|---|---|
| 11 | *1929*<br>Nov. 15 | 7.410 | 7.36 | 0.05 | Kept at 22° for 10 min. to permit clotting, then centrifuged. |
|  |  | 7.385 | 7.31 | 0.08 | Kept at 28° for 25 min., then centrifuged. |
| 13 | Nov. 22 | 7.369 | 7.30 | 0.07 | Kept at 22° for 10 min. to permit clotting, then centrifuged. |
|  |  | 7.040 | 6.92 | 0.12 | Kept at 30° for 1¾ hrs.; centrifuged but serum not drawn from clot for 2 hrs. |
| 12 | Dec. 13 | 7.384 | 7.32 | 0.06 | Kept at 25° for 10 min. to permit clotting, then centrifuged. |
|  |  | 7.355 | 7.26 | 0.10 | Kept at 29° for 18 min., at 25° for 1 hr., then centrifuged. |
| 14 | *1930*<br>Jan. 11 | 7.262 | 7.23 | 0.03 | Kept at 25° for 39 min., then centrifuged. |
|  |  | 7.257 | 7.22 | 0.04 | Kept at 30° for 18 min., at 25° for 1½ hrs., then centrifuged. |
| 11 | Jan. 16 | 7.368 | 7.31 | 0.06 | Kept at 20° for 10 min. to permit clotting, then centrifuged. |
|  |  | 7.335 | 7.26 | 0.07 | Kept at 25° for 1 hr., then centrifuged. |
| 15 | Jan. 24 | 7.358 | 7.34 | 0.02 | Kept at 20° for 10 min. to permit clotting, then centrifuged. |
|  |  | 7.264 | 7.25 | 0.01 | Kept at 38° for 3 hrs., then centrifuged. |
| 19 | Mar. 10 | 7.385 | 7.34 | 0.05 | Kept on ice for 3 hrs., then centrifuged. |
|  |  | 7.288 | 7.21 | 0.08 | Kept at 38° for 3 hrs., then centrifuged. |

pH of the sera which had been in contact with the clot for longer periods of time. Whereas in the case of Dog 13 the greatest increase in acidity seemed to be associated with the greatest rise in the *Q* correction, yet in Dog 15 an increase in acidity of 0.1 pH occurred with no change in the *Q* correction. In Dogs 11, 12, and 11 (repeated) increases in the *Q* correction were accompanied by only small increases in acidity. Thus, there appears to be little correlation between glycolysis and the increase in the *Q* correction. It is interesting to note that three experiments showed no change in the *Q* correction, even though in one of these, Dog 15, the blood stood 3 hours at 38° before centrifuging.

Thus it appears that serum for pH work with the quinhydrone electrode should be separated as soon as possible from the corpuscles. While blood is usually centrifuged promptly, these results suggest that the equilibration of blood samples outside the body at 38° requiring considerable lengths of time may cause changes in the serum which may influence the *Q* correction.

TABLE VII.

*Comparative pH Determination on Dog Serum and Plasma at 38° by Hydrogen and Quinhydrone Electrodes.*

| | $pH_{H_2}$ | $pH_Q$ | $pH_{H_2} - pH_Q$ |
|---|---|---|---|
| Serum | 7.381* | 7.36 | +0.02 |
| Plasma† | 7.422 | 7.39 | +0.03 |
| Serum | 7.384 | 7.35 | +0.03 |
| Plasma | 7.390 | 7.36 | +0.03 |
| Serum | 7.353 | 7.34 | +0 01 |
| Plasma | 7.357 | 7.34 | +0 02 |

* The animal struggled during the collection of this serum sample.

† Anticoagulant: 0.4 per cent mixture 3:1 potassium oxalate-sodium fluoride.

## *Serum and Plasma.*

The experiments listed in Table VII were performed to determine whether the *Q* corrections for plasma are the same as for serum. Such was found to be the case.

Incidentally, the last two experiments on the pH of serum and plasma lend further substantiation to the observations of Ross (18) and Bennett (19), who found no difference in pH between serum and plasma. This is not, however, in agreement with Kugelmass

(20) nor with Stuber and Lang (21), who found that clotting produced an alkalinity due to absorption of hydrogen ions by the clot. But it must be pointed out that these latter investigators studied the mechanism of clotting with more or less purified mixtures. Their findings may not necessarily be strictly applicable to such complex systems as whole blood.

### *First Acid Change.*

Recently, Havard and Kerridge (22) have published a series of determinations of the pH of human whole blood with the glass electrode. They made the very interesting and important observation that blood, after being drawn, became 0.02 to 0.05 pH more acid within 2 to 8 minutes if kept at 38°. At lower temperatures, the change came on more slowly: in 6 to 14 minutes at 28°; in 1½ hours at 18°. This acid change seemed not to be associated with clotting, since it occurred both before and after the blood clotted. Immediate chilling prevented the acid change altogether. The addition of NaF, while effective in preventing the later acid change observed by Evans (23), was non-effective. The acid change did not occur in separated serum or plasma, but did occur when corpuscles were added. Thus it appeared that the first acid change was due to an acid substance from the corpuscles. Preliminary experiments indicated that this acid substance was probably not lactic acid.

In view of our experience with blood kept at high temperatures before centrifuging, there was the possibility that the high $Q$ corrections thus obtained might reflect the presence of disturbing substances produced from the corpuscles. With this in mind, a series of determinations was made to test the findings of Havard and Kerridge, with the hydrogen and quinhydrone electrodes, and to see whether there was any correlation between the first acid change and high $Q$ corrections. It was found desirable in these experiments to use anticoagulants, since the low temperatures prolonged the clotting time of blood so greatly that the serum often clotted after centrifuging. One sample of blood was drawn into chilled centrifuge tubes kept in an ice bath; another sample was drawn into warm tubes kept at 38°. After standing at 30–35° for from 10 to 15 minutes, the warm sample was chilled, and both samples were centrifuged in the cold room. By this procedure there was obtained a plasma sample from blood before and after the supposed first acid change. In every case, the pH of the plasma from the chilled blood was higher than that from the warmed blood. The data are given in Table VIII.

Examination of the *Q* corrections shows that the appearance of the first acid change has left them unaltered. The relatively shorter exposures of the blood to high temperatures before centrifuging and the use of anticoagulants seem to offer a possible explanation why in this series of experiments the *Q* corrections in the warm samples did not rise as they did in the earlier experiments of Table IV.

TABLE VIII.

*First Acid Change in Shed Blood.*

| $pH_{H_2}$ | First acid change. | $pH_Q$ | $pH_{H_2} - pH_Q$ | Treatment of blood. |
|---|---|---|---|---|
| 7.432 | | 7.44 | −0.01 | Chilled and centrifuged at 10°. |
| 7.392 | 0.040 | 7.41 | −0.02 | Kept 13 min. at 36°, then chilled and centrifuged at 10°. |
| 7.452 | | 7.44 | +0.01 | Chilled and centrifuged at 10°. |
| 7.432 | 0.020 | 7.41 | +0.02 | Kept 12 min. at 33°, then chilled and centrifuged at 10°. |
| 7.480 | | 7.47 | +0.01 | Chilled and centrifuged at 10°. |
| 7.448 | 0.032 | 7.44 | +0.01 | Kept 15 min. at 30°, then chilled and centrifuged at 10°. |

### *Quinhydrone Determinations at 20°.*

Comparisons by other investigators of the pH of serum at 20° by the hydrogen and quinhydrone electrodes have been confined largely to human material. A search through the literature has revealed only two references dealing with comparative determinations on dog serum at 20°. Cullen and Earle (9) report two experiments where the quinhydrone electrode averaged 0.09 pH more acid than the hydrogen electrode value. This is somewhat higher than the correction factor they found for human serum (0.06). They state, however, that in view of the discrepancies of the colorimetric method which occur in dog serum, more data must be accumulated before such a *Q* correction can be accurately known. The average of four values on one sample of dog plasma from Vellinger and Roche's (24) paper, gives a *Q* correction of 0.11 pH.

In order to accumulate additional data on the *Q* correction of dog serum at 20° and especially to enable us to compare our results with the data in the literature at room temperature, the pH of a series of dog sera was determined by the hydrogen and quinhydrone electrodes at 20°. It was found that the drift of the quinhydrone electrode was considerably less at 20°. Except in a few cases, the drift was not apparent until about 30 seconds after contact was made, thereafter, a drift toward more acid pH slowly began. As far as can be gathered from the work of Cullen and Earle, the drifts observed by them at 20° were similar to ours. Table IX shows the results obtained. The average *Q* correction was +0.03, which is in exact agreement with our value at 38°. Save for one quinhydrone result obtained 24 hours after hemorrhage, the deviations from this mean *Q* correction were somewhat less than those found at 38°. This may, however, not be significant in view of the comparatively small number of determinations made. It is interesting to note that except for the one case already mentioned the *Q* corrections obtained were very much less than either those of Cullen and Earle or Vellinger and Roche.

TABLE IX.

*Comparison of pH of Normal Dog Serum at 20° by Hydrogen and Quinhydrone Electrodes.*

| Dog No. | Date. | Hydrogen electrode. | | Quinhydrone electrode. | | $pH_{H_2} - pH_Q$ |
|---|---|---|---|---|---|---|
| | | Temperature. | pH | Temperature. | pH | |
| | *1930* | *°C.* | | *°C.* | | |
| 11 | Mar. 19 | 20.5 | 7.522 | 20.5 | 7.51 | +0.01 |
| 12 | " 22 | 20.5 | 7.598 | 19.5 | 7.59 | +0.01 |
| 16 | " 27 | 20.0 | 7.582 | 19.5 | 7.54 | +0.04 |
| 15 | " 24 | 21.0 | 7.578 | 21.0 | 7.54 | +0.04 |
| 17 | " 28 | 20.0 | 7.562 | 19.5 | 7.54 | +0.02 |

## *Temperature Coefficient.*

In four experiments given in Table X, simultaneous determinations were made at 20° and 38° on serum. From these data, the temperature coefficient of serum could be calculated on the assumption that between 20–38°, the change in pH per degree was constant. The particular fact to be brought out is that this change appears to be the same for the hydrogen and quinhydrone electrodes, a conclusion which necessarily follows from the observations made concerning the $Q$ correction at 20° and at 38°. This coefficient, averaging 0.013 pH per degree in the four cases tabulated, agrees with the findings of Cullen, Keeler, and Robinson (25). A calculation from four of their experiments with the hydrogen electrode at 20° and 38° gives an average coefficient of 0.012 pH. The experiment on Dog 17 was designed to show whether experimental variation of the protein concentration of serum would exercise any change on the temperature coefficient of the pH as measured with the hydrogen and quinhydrone electrodes. On theoretical grounds, a lowering of the temperature coefficient might be expected 24 hours after hemorrhage, when the serum proteins were probably somewhat diluted. Our experiment, however, does not show any decisive lowering of the temperature coefficient of the pH of the serum after hemorrhage, at least with the hydrogen electrode, and in the absence of additional experiments, the result with the quinhydrone electrode must await explanation.

TABLE X.

*Temperature Coefficient of pH of Dog Serum As Determined by Hydrogen and Quinhydrone Electrodes.*

| Dog No. | Date. | Hydrogen electrode. | | | Quinhydrone electrode. | | | $pH_{H_2} - pH_Q$ |
|---|---|---|---|---|---|---|---|---|
| | | Temperature. | pH | Coefficient. | Temperature. | pH | Coefficient. | |
| | *1930* | °*C.* | | $\frac{dpH}{dt}$ | °*C.* | | $\frac{dpH}{dt}$ | |
| 15 | Jan. 29 | 38 | 7.369 | | 38 | 7.35 | | +0.02 |
| | | 22 | 7.610 | 0.015 | 21 | 7.58 | 0.014 | +0.03 |
| 11 | Apr. 4 | 38 | 7.365 | | 38 | 7.31 | | +0.06 |
| | | 21 | 7.592 | 0.013 | 20 | 7.55 | 0.014 | +0.04 |
| 17 | Apr. 17 | 38 | 7.339 | | 38 | 7.31 | | +0.03 |
| | | 20.3 | 7.582 | 0.014 | 20.2 | 7.54 | 0.013 | +0.04 |
| 17* | Apr. 18 | 38 | 7.395 | | 38 | 7.35 | | +0.05 |
| | | 20.1 | 7.623 | 0.013 | 20 | 7.55 | 0.011 | +0 07 |

* Sample taken 24 hours after a 3.7 per cent hemorrhage.

### *Abnormal Sera.*

During the course of the work, several serum samples were obtained from dogs which did not appear to be normal. Two samples were taken from dogs with biliary fistulas, and had the characteristic deep yellow color. These gave *Q* corrections of +0.07 and +0.05. It may be that the presence of excessive amounts of biliary substances in the serum caused the slightly higher *Q* correction, such as was noted in the one case. Two other samples of serum were obtained from dogs with symptoms of distemper. Here the *Q* correction averaged +0.05 pH which is within the normal range. One sample of serum obtained from a dog after acid injection was very much hemolyzed. It was expected, in view of the observations of the alkaline drift of the quinhydrone electrode in whole blood (16, 26), that the drift in this particular sample would be modified. This was not the case. The drift was of the same order and in the same direction as with unhemolyzed serum. This was also our experience in several cases where only slight hemolysis occurred. Mislowitzer (27) states specifically that the presence of hemoglobin in serum does not affect the drift of the quinhydrone electrode. A buffer solution to which he added hemoglobin did not produce any drift in the quinhydrone electrode towards more alkaline values. We have, however, to explain the fact that in our hemolyzed serum, the *Q* correction was negative instead of positive (−0.05). Since diluted whole blood, as Mislowitzer and also Liu (28) found, gives quinhydrone readings which average 0.02 pH more alkaline than the hydrogen electrode, this observation may explain the above negative correction. Nevertheless, in several samples of serum which were only slightly hemolyzed no significant changes in the *Q* corrections could be observed.

## DISCUSSION.

The following facts were gleaned from a study of the distribution curve of the *Q* corrections of 65 determinations, which include all the serum and plasma, normal and abnormal. Out of this number it seems reasonable to eliminate the very small number of extreme variations of the *Q* corrections, especially when one remembers that the causes which are responsible for their occurrence appear to be known and can, therefore, be avoided. When this is done, the limits of the *Q* correction are from −0.02 to +0.06, a spread of 0.08 pH. If the average *Q* correction is taken as +0.03, then within these limits 93 per cent of all the determinations checks results with the hydrogen electrode within ±0.03 pH and 83 per cent within ±0.02. The variations in the *Q* correction do not seem to have any relation to the pH of the serum.

A sufficiently large number of comparative determinations of the pH of dog serum and plasma have been made under a variety of experimental conditions to justify an evaluation of the quinhydrone method. Since the electrode represents an oxidation-reduction system, undesirable side reactions with some of the constituents of the solutions in which it is to be used may take place. This may produce drifting potentials. In serum at 38° the drift is so great that it is necessary to resort to extrapolation to obtain suitable results. On theoretical grounds, such a procedure is open to serious criticism; on practical grounds, the increased complexity of technique and consequent possibility of error are important considerations. The method is empirical; the accuracy of its results must largely serve as its justification. But it is believed that a degree of accuracy is attainable which is adequate for much of the present biochemical research. The drifts are far less at 20°, and the determination is, therefore, simpler, but at the present time we hesitate to assume that the temperature correction which must be applied to obtain values for 38° is constant for all serum.

This study indicates that the method of determining pH by the quinhydrone electrode is subject to fewer errors than the colorimetric method. It possesses no inherent subjective errors, and in general gives more reliable data under experimental conditions. While less accurate than the hydrogen electrode, the small amounts of material needed for the quinhydrone electrode determination, and the rapidity with which determinations may be made, make the quinhydrone electrode a decidedly useful instrument for studying the pH of blood and other tissue fluids.

## SUMMARY.

1. A comparative study of the pH of normal dog serum and plasma at 38° by the hydrogen and quinhydrone electrodes has been made. On the average, the quinhydrone electrode reads 0.03 pH more acid than the hydrogen electrode. Under experi-

mental conditions this difference has been found to remain sufficiently constant to inspire confidence in the reliability of the method. Determinations of pH with the quinhydrone electrode can be rapidly made and require but small amounts of fluid.

2. The occurrence of the first acid change of shed blood, discovered by Havard and Kerridge, has been substantiated both with the hydrogen and quinhydrone electrodes.

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# THE METABOLISM OF THE PHOSPHOLIPIDS.

## II. THE INFLUENCE OF GROWTH ON THE PHOSPHOLIPID (AND CHOLESTEROL) CONTENT OF THE WHITE RAT.

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### INTRODUCTION.

In the course of an investigation of the influence of the character and the amount of the fat of the food on the composition of the lipids, especially the phospholipids, of the white rat, data have been obtained on a series of rats ranging in weight from about 30 up to about 200 gm. It soon became apparent that the smaller the rat the higher was the percentage content of phospholipid fatty acids. A brief search of the literature having failed to reveal any such intimate and comprehensive investigation of the change in the phospholipid content of animal tissues with age, as the phenomenon would seem to justify, the original investigation was extended to include some analyses of suckling rats in order to cover the complete range from birth to adult age.

Mayer and Schaeffer (1914) were probably the first to observe the change in the phospholipid content of animals during growth. Their conclusion was that the percentage content of lipid phosphorus in rats increases sharply following birth and then remains practically constant throughout life. However, their data applied chiefly to rats during the first 3 weeks of postnatal life and, as will be pointed out later, it is altogether likely that the increase in the phospholipid content during this period is entirely due to the rapid decrease in the water content of the tissues. Recently, Javillier, Allaire, and Rousseau (1927) have observed an increase in the lipid phosphorus content of mice during the first 21 days of postnatal life. Furthermore, these authors determined the water content and, from the values obtained, calculated that the percentage content of lipid phosphorus in the dry tissues also increases after birth. On the other hand, Robertson (1916) determined the content of alcohol-soluble phosphorus in mice of various ages and found a steady decrease, commencing at birth and continuing throughout life. In the light of our own results and those of Mayer and Schaeffer, and Javillier *et al.* it is difficult indeed to understand why Robertson did not find a lower content of alcohol-soluble phosphorus in mice at birth than at 14 or 35 days of age.

At best our own data are far from ideal for the purpose in hand but they are sufficiently consistent to permit some general conclusions concerning the influence of growth on the phospholipid content of animal tissues.

Graphical analysis of the data has revealed: (1) that there is a progressive decrease in the water content of the rat after birth, the most rapid period of dehydration being from birth to weaning age, in which time 50 per cent of the total loss in the first 100 days of life occurs; (2) that the percentage content of phospholipid fatty acids in the dry tissues decreases rapidly during the first 3 months of life; (3) that in consequence of the rapid decrease in water content during the first 3 weeks of postnatal life, the percentage content of phospholipid fatty acids in the moist tissue increases rapidly after birth, attains a maximum in about 3 weeks, and then declines throughout the following 13 weeks of life; (4) that the phospholipid : cholesterol ratio in the entire rat remains practically constant throughout the first 100 days of life.

## EXPERIMENTAL.

The population of rats upon which the following data have been obtained was raised for the most part under quite varied circumstances. Some of the rats were raised in stock cages with half a dozen, more or less, other rats of about the same age; others were raised in individual metabolism cages made throughout of No. 2 mesh wire screen. The most important variant was the diet. Some of the rats were fed on a diet of kitchen scraps, while the great majority were raised, after weaning, on a ration consisting of alcohol-extracted casein, cane sugar, McCollum's Salt Mixture 185,[1] ether-extracted dried yeast, and Oscodal,[2] generally supplemented

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[1] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

[2] A cod liver oil concentrate containing vitamins A and D, kindly supplied by Dr. H. E. Dubin of the H. A. Metz Laboratories, Incorporated.

with different fats (cod liver oil, lard, olive oil, coconut oil, linseed oil) in amounts ranging from 2.5 to 40 per cent of the total calories. In all cases food and water were provided *ad libitum.*

The analysis of the lipids in the whole rats was carried out as follows: After the live weight had been obtained, the rat was stunned by a blow on the head and immediately the entire animal was hashed in a meat grinder. The hashed tissue remaining in the grinder was removed, the total lot thoroughly mixed, and again passed through the grinder. A 50 gm. sample of the well mixed hashed tissue was weighed out, and then stirred up in about 3 volumes of 95 per cent alcohol. After a few minutes standing the hashed tissue and alcohol were poured into the cloth sack of the hot alcohol extractor in use in this laboratory. The extraction was continued for 3 hours, the alcoholic extract being replaced by fresh alcohol and the tissue well stirred at the end of each hour. The combined alcoholic extracts were distilled practically to dryness on a water bath under reduced pressure. The pasty residue was taken up in moist ether, the ether extract (which in most cases was quite turbid) concentrated, and then washed with ether into acetone in a 100 cc. centrifuge tube. To facilitate complete precipitation of the phospholipids 2 cc. of a saturated solution of $MgCl_2$ in alcohol were added to the mixture of acetone and ether in the centrifuge tube. After centrifugalization the supernatant acetone and ether solution of the neutral fat and cholesterol was poured off into a flask, and the acetone-insoluble lipids, consisting mainly of the phospholipids, were rubbed up in fresh acetone. To insure against contamination of the phospholipids by neutral fat, the phospholipid fraction was redissolved in ether and again precipitated with acetone and $MgCl_2$. The phospholipid fraction was redissolved in ether—the ether solution was always quite turbid and contained a considerable amount of suspended material, probably non-lipid in nature—and transferred to a saponification flask. After evaporation of the ether, the phospholipids were saponified with NaOH in 50 per cent alcohol for 3 to 4 hours. The alcoholic solution was cooled and made acid to Congo red paper with concentrated HCl. The fatty acids were extracted with petroleum ether, transferred to a volumetric flask, and a suitable aliquot taken for weight and iodine number determination.[3]

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[3] The data on the iodine numbers of the phospholipid and neutral fat fatty acids will be published in a separate paper.

The acetone-ether solution of the neutral fat and unsaponifiable substances was distilled to a small volume, rinsed into a separatory funnel with petroleum ether, and washed with water. The petroleum ether solution was concentrated and made up to volume in a volumetric flask. An aliquot of ⅕ or ⅖ was taken for weight and another was pipetted into a saponification flask, and, after evaporation of the petroleum ether, saponified with equal volumes of N sodium ethylate and absolute alcohol. After several hours of boiling under a reflux condenser, an equal volume of water was added and boiling continued for 2 to 3 hours longer. Then the soap solution was cooled, transferred to a separatory funnel, shaken up with petroleum ether, and allowed to stand overnight. The next day the soap solution was drawn off, the petroleum ether extract washed with 50 per cent alcohol, and again separated. The petroleum ether extract was distilled and the residue of unsaponifiable matter was dried and weighed. After acidification of the soap solution, the fatty acids were extracted with petroleum ether, made up to volume, and an aliquot taken for weight and iodine number determination.[3]

The tissue residue remaining after the 3 hours extraction with hot alcohol was dried in the air and weighed. This weight was found to be a fairly accurate measure of the water-free, lipid-free tissue solids.

Since neutral fat is probably to be regarded as inert deposit material, a calculation has been made of the fat-free moist weight of all rats. However, this weight is still an inaccurate measure of the active protoplasm since it includes the weight of the skeleton, teeth, hair, and the other relatively inert structural elements of the body. A correction for the weight of the bony skeleton could have been made by using the data given by Donaldson (1924, p. 188), but there seemed to be no particular advantage in doing so. A correction for the weight of the deposit fat was quite essential since the fat content of animals of the same age varied greatly because of the marked difference in the fat-producing quality of the various diets used.

From the data obtained, calculations have been made of the percentage content of phospholipid fatty acids, neutral fat, and unsaponifiable material in terms of the moist fat-free weight and the dry extracted weight of the tissue.

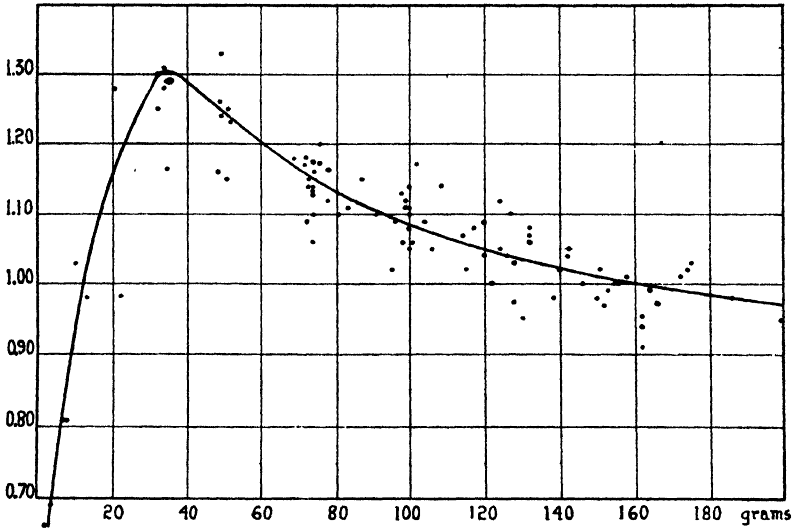

FIG. 1. Chart showing the change in the content of phospholipid fatty acids in the moist fat-free tissues of the rat with increase in body weight. Gm. of phospholipid fatty acids per 100 gm. of moist fat-free tissue are plotted on the ordinate and body weight on the abscissa. In this and the succeeding figures the curves have been placed purely by inspection.

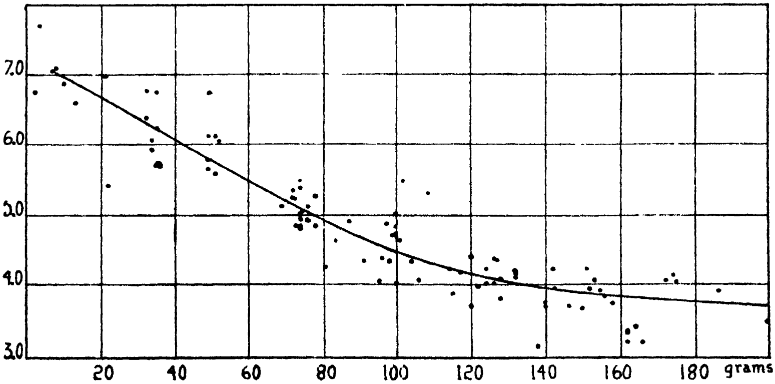

FIG. 2. Chart showing the change in the content of phospholipid fatty acids in relation to the weight of the dry extracted tissues of the rat with increase in body weight. Gm. of phospholipid fatty acids per 100 gm. of dry extracted tissue are plotted on the ordinate, body weight on the abscissa.

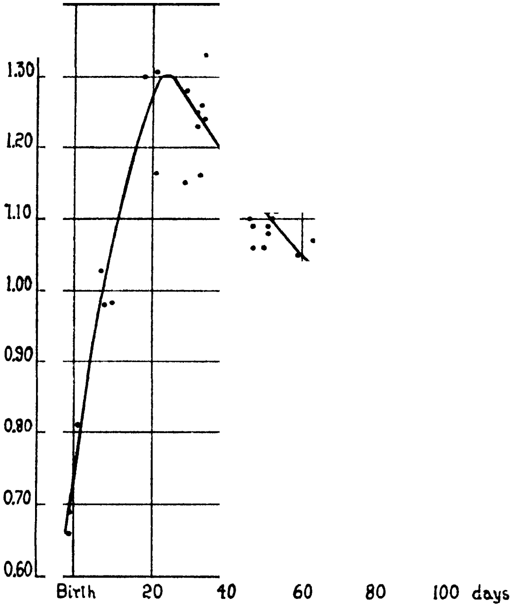

FIG. 3. Chart showing the change in the content of phospholipid fatty acids in the moist fat-free tissue with increase in age. Gm. of phospholipid fatty acids per 100 gm. of moist fat-free tissue are plotted on the ordinate, postnatal age on the abscissa.

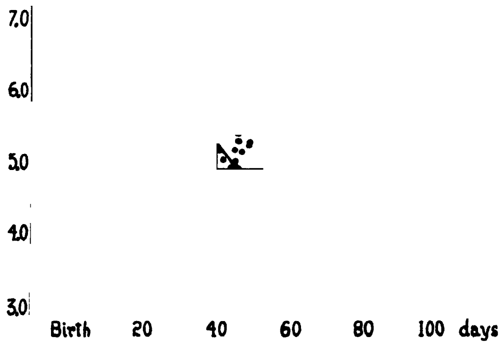

FIG. 4. Chart showing the change in the content of phospholipid fatty acids in relation to the weight of the dry extracted tissues with increase in age. Gm. of phospholipid fatty acids per 100 gm. of dry extracted tissue are plotted on the ordinate, postnatal age in days on the abscissa.

When the live weight of the rat taken was less than 50 gm., all of the hashed tissue was carefully removed from the grinder by rinsing with alcohol and included in the sample for extraction. Furthermore, in the cases of the very small rats weighing less than

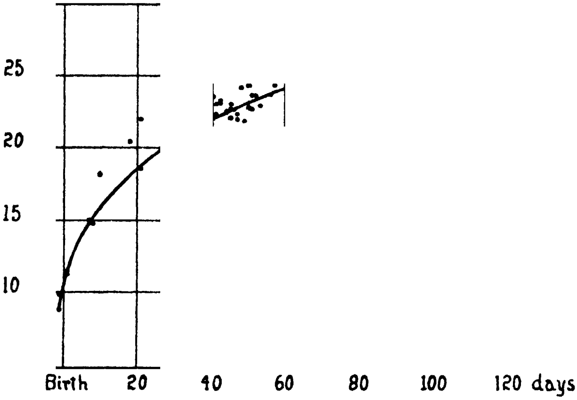

Fig. 5. Chart showing the change in the water content of the tissues of the rat with increase in age. Gm. of dry extracted tissue per 100 gm. of moist fat-free tissue are plotted on the ordinate, age on the abscissa.

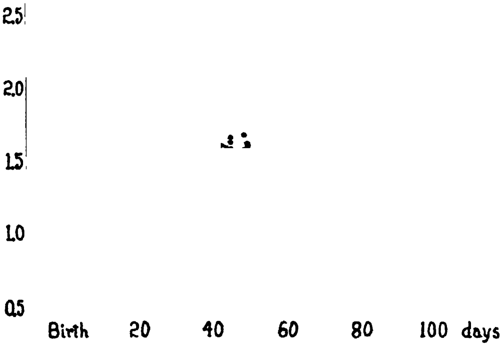

Fig. 6. Chart showing the change in the content of unsaponifiable material in relation to the weight of the dry extracted tissues of the rat with increase in age. Gm. of unsaponifiable material per 100 gm. of dry extracted tissue are plotted on the ordinate, age in days on the abscissa.

20 gm., the hashed tissue was further ground in a mortar with a weighed amount of sand. The paste of sand and tissue, as well as the traces of tissue adhering to the grinder, were rinsed with alcohol into a weighed Schleicher and Schüll extraction thimble. The extraction was carried out in the ordinary hot alcohol extractor, the alcohol being changed and the tissue and sand rubbed up with alcohol in a mortar at the end of each hour. After the extraction was completed, the shell, tissue residue, and sand were dried and weighed; since the weight of both the shell and sand was known, the weight of dry extracted tissue could be calculated.

The completeness of the hot alcohol extraction of the lipids from the hashed rat tissue was tested in two ways; first, by extracting the tissue residues with ether in a Soxhlet apparatus for 6 hours; second, by saponifying the residues with strong alkali, extracting the acidified solution with ether, evaporating to dryness, and extracting the ether residue with petroleum ether. The residues of two batches of very young rats treated by the first method yielded less than 1 mg. of ether residue in either case, while the combined residues from several rats treated by the second method yielded fatty material amounting to, on the average, 0.56 per cent of the total lipids extracted.

### *Results.*

The data obtained have been plotted in the form of graphs to show the change in the content of water, of phospholipid fatty acids, and of unsaponifiable material with increase in body weight and with increase in age. There is quite a considerable scattering of the points on the graphs (Figs. 1–6), a fact scarcely to be wondered at in view of the heterogeneity of the population with respect to diet and, therefore, the rate of growth. Nevertheless, the curves leave little room for doubt as to the general effect of growth on the content of phospholipid and of unsaponifiable material in the white rat.

## DISCUSSION.

Since the phospholipid content of such organs as the brain, heart, liver, and kidneys is notably higher than that of others, particularly the muscles, one is led to wonder if a shift in the relative proportions of the various organs of the body might not be responsible for the apparent decrease in the phospholipid content of the rat during growth. Obviously an increase in the relative proportion of muscle or bone would tend to decrease the phospholipid content of the entire animal.

TABLE I.

*Percentage Weight of Brain and Viscera in Rats of Various Sizes.*

| Body weight. | Brain. | | Viscera.* | |
|---|---|---|---|---|
| | Per cent of body weight. | Relative per cent. | Per cent of body weight. | Relative per cent. |
| *gm.* | | | | |
| 4.9 | 4.61 | 1.00 | 10.89 | 1.00 |
| 25.0 | 5.03 | 1.09 | 19.23 | 1.76 |
| 50.0 | 2.97 | 0.64 | 17.74 | 1.64 |
| 100.0 | 1.68 | 0.36 | 14.94 | 1.38 |
| 200.0 | 0.93 | 0.20 | 12.39 | 1.14 |
| 300.0 | 0.66 | 0.14 | 11.19 | 1.03 |
| 400.0 | 0.51 | 0.11 | 10.51 | 0.97 |

* Viscera includes heart, hypophysis, intestines, kidneys, liver, lungs, pancreas, spleen, stomach, submaxillaries, suprarenals, and thyroid.

TABLE II.

*Content of Phospholipid Fatty Acids in the Carcasses* of Rats at Various Ages.*

| Age. | Body weight. | Weight of carcass in relation to body weight. | Weight of phospholipid fatty acids per 100 gm. of: | |
|---|---|---|---|---|
| | | | Moist fat-free tissue. | Dry extracted tissue. |
| *days* | *gm.* | *per cent* | *gm.* | *gm.* |
| 28 | 34 | 34.8 | 1.20 | 6.26 |
| 41 | 76 | 38.3 | 1.12 | 4.64 |
| 43 | 80 | 36.8 | 1.13 | 4.59 |
| 51 | 76 | 36.0 | 1.17 | 4.79 |
| 61 | 130 | 40.8 | 1.06 | 4.05 |
| 59 | 138 | 43.1 | 1.01 | 3.93 |
| 73 | 156 | 40.2 | 0.98 | 3.90 |
| 83 | 164 | 40.9 | 0.97 | 3.84 |
| 96 | 172 | 40.4 | 0.94 | 3.69 |
| 107 | 166 | 41.4 | 1.06 | 3.98 |

* The carcass comprises skeletal muscle and bone; the skin, head, tail, feet, and viscera being discarded.

The marked change in the percentage weight of the brain and viscera during the growth of the rat as shown by Table I (compiled from Donaldson (1924)) illustrates how important a factor the relative decrease in the weight of such phospholipid-rich tissues as the brain, heart, liver, and kidneys may be in causing the observed decrease in the phospholipid content of the entire rat. However, the data which have been obtained on the carcasses of rats of various ages (Table II) show that in the carcass as well as in the entire animal there is a decrease in the content of phospholipid fatty acids with increase in body weight. Unfortunately the interpretation of these data is also complicated in that the carcasses include the skeleton as well as the musculature and there is no saying how much of the phospholipid is contributed by the bone marrow; nevertheless it seems probable that these data indicate an actual decrease in the phospholipid content of the skeletal muscles during growth. Since the percentage weight of the skeleton decreases during growth (Donaldson, 1924), the inclusion of the bone with the soft tissues tends to diminish rather than to magnify the effect of growth on the phospholipid content of both the entire animal and the carcass. Koch and Koch (1913) have shown that the phospholipid content of rat brains, expressed in relation to the dry solids, increases from birth to about the age of 3 weeks and thereafter remains practically constant.

That the water content of animal tissues decreases throughout life has been observed by a number of investigators (Moulton, 1923). This fact is illustrated by Fig. 5 which shows the influence of age on the percentage content of dry lipid-free solids. The water content may be assumed to be approximately the difference between the moist fat-free weight and the dry extracted weight plus the phospholipid since the data calculated in this manner show good agreement with those obtained by Hatai (cited by Moulton (1923)).

The rapid decrease in the water content of the rat during the first 3 weeks of postnatal life is of especial interest in that it seems to be entirely responsible for the peculiar conformation of the curves in Figs. 1 and 3. These curves apparently show a rapid increase in the phospholipid content of the moist tissue during the first 3 weeks of life, although actually the phospholipid content of the tissue solids is decreasing as is shown by Figs. 2 and 4.

In Fig. 6 the weights of the unsaponifiable material per 100 gm. of dry extracted tissue have been plotted against the age of the rats. Despite the very considerable scattering of the points—for which there seems to be no satisfactory explanation—it is believed that the curve which has been drawn is reasonably representative and may well be taken as a measure of the influence of age on the content of unsaponifiable material in the white rat.

In view of the importance which is frequently attached to the phospholipid : cholesterol ratio it is of interest to determine whether or not the process of growth, which has a marked influence on the content of phospholipid and unsaponifiable material in the entire rat, also has an effect upon the ratio of these substances to one another. Accordingly the ratios phospholipid fatty acids: unsaponifiable material have been calculated for every 10 day interval from birth to 100 days of age, from the values given by the curves in Figs. 4 and 6. Since the ratios thus calculated vary, quite independently of age, between the rather narrow limits of 3.0 to 3.3, it seems probable that growth influences the content of phospholipid and unsaponifiable material in the rat to about the same extent, the ratio of the two substances thereby remaining constant.

Since the total unsaponifiable material includes, besides cholesterol, other substances about which very little is known, it seemed desirable to determine the percentage content of cholesterol in the unsaponifiable material. This can easily be done by the Bloor colorimetric method (1916) which is based upon the Liebermann-Burchard reaction for cholesterol. The cholesterol content of the unsaponifiable material from twenty-four rats, ranging in age from newborn to 105 days, fell between 57.1 and 75.5 per cent, with an average value of 67.1 ±5.6 per cent. With the use of this average value of 67.1 per cent of cholesterol in the unsaponifiable material, and the value 66 per cent for the percentage weight of fatty acids in the phospholipid, the average phospholipid : cholesterol ratio for the entire body of the white rat was found to be 7.0.

At the present time it would seem to be rather difficult to arrive at any satisfactory conclusion as to the physiological significance of the marked decrease in the phospholipid content of the rat with increase in body weight. An extensive study of the phospholipid content of various organs of the beef led Bloor (1926, 1927, 1928) to conclude that the phospholipid content of a tissue is a function of its physiological activity. On this basis, the very considerable decrease in the phospholipid content of rats during the first 3 months of postnatal life would indicate a progressive decline in the physiological activity during this period. However, in so far as muscular activity is concerned, there seems to be a lack of agreement between the phospholipid content of the skeletal muscles of the rat and their degree of activity since, according to Slonaker (1907), the activity of rats increases with age up to 87 to 120 days whereas during this period the phospholipid content is steadily decreasing (Table II).

With respect to the cause of the decrease in phospholipid content with increase in body weight, the shape of the curves in Figs. 1 to 4 indicates that the period of most rapid decline in phospholipid content coincides with the period of most rapid growth. This fact suggests that there may be a certain measure of independence in the rates of synthesis of phospholipid and of the other solid constituents of the tissues. In this connection the case of a young rat which grew at an unusually rapid rate may be of some significance; the phospholipid content of this young rat was found to be much below the normal value for rats of the same age or of the same weight whereas the actual weight of phospholipid was approximately normal for its age. Of interest also are the data on a rat which for some unknown reason maintained a constant weight of around 36 gm. for 8 weeks. The data for this abnormal rat in the charts have been encircled by a line. It may be seen that the content of phospholipid fatty acids corresponds quite closely to that of normal rats of the same size but much younger. This fact is especially interesting since, according to Mendel and Judson (1916), the water content of rats stunted by deficient diets is typical of their age rather than of their body weight.

## SUMMARY.

A study of the content of phospholipid fatty acids and unsaponifiable material in the entire bodies of white rats during the first 4 months of life has shown:

1. That the phospholipid content, when expressed in relation to the tissue solids, decreases rapidly after birth, the period of most rapid decline in the phospholipid content coinciding with the period of most rapid growth.

2. On account of the rapid decrease in the water content of the tissues of the rat as a whole during the first few weeks of postnatal life, the phospholipid content of the moist fat-free tissues increases rapidly after birth, reaches a miximum in about 3 weeks, and then declines throughout the remainder of the period studied.

3. The ratio phospholipid fatty acids: unsaponifiable material remains practically constant during the first 3 months of life.

The author is indebted to Professor W. R. Bloor for advice and criticism throughout the progress of this work.

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# THE APPARENT DISSOCIATION CONSTANTS OF DIIODOTYROSINE, ITS HEAT OF SOLUTION, AND ITS APPARENT HEAT OF IONIZATION.*

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The present work is a continuation of the systematic survey of the apparent dissociation constants of the amino acids which has been in progress in this laboratory for some time (1). We are reporting not only the apparent dissociation constants of diiodotyrosine but also its apparent heat of ionization and its heat of solution. This amino acid which was discovered by Drechsel (2) in 1896 has recently assumed a rôle of added importance due to its presence in the thyroid gland (3).

Our product was prepared by iodizing *l*-tyrosine in accordance with the procedure recommended by Henze (4). It yielded, within experimental limits, theoretical values for iodine. Another synthesis was carried out according to the method of Harington (5). For the purpose of estimating its apparent dissociation constants we determined the solubility of diiodotyrosine in solutions containing varying amounts of sodium hydroxide or of hydrochloric acid, a procedure which was previously employed by Sano (6) and shown to be theoretically justifiable by Hitchcock (7). The solubility of diiodotyrosine was determined by placing the amino acid and the dilute acid or base to be used in an inverted Pyrex T-tube of about 100 ml. capacity. The lower end of the T-tube was somewhat curved to facilitate the withdrawal of fluid. Except for the determinations which were carried out at

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0° the tubes were immersed in an oil bath kept within ± 0.05° of the given temperature. For the 0° work an ice water bath was employed. The tubes were shaken for a period of 24 to 36 hours. The clear fluid was obtained by pressure filtration through a glass tube containing a layer of cotton and a layer of asbestos. The amount of diiodotyrosine which was dissolved was estimated on the basis of the iodine content. The procedure of Kelly and Husband (8) was in its essential features followed in estimating iodine.

The hydrogen ion activity at 25° was determined with the aid of the quinhydrone electrode. The accuracy of this method was checked against the hydrogen electrode. At 0° and 40° the hydrogen ion activities were determined with the aid of the hydrogen electrode. We did not employ the quinhydrone electrode at these temperatures since it has not been studied with any degree of accuracy. The technique described by Kirk and Schmidt (9) was in its essential features followed for the estimation of hydrogen ion activity. The values for the E.M.F. referred to the 1.0 N calomel half-cell when connected with a hydrogen electrode immersed in a solution of hydrochloric acid having an activity value of unity at 0° and 40° respectively were determined in a manner similar to that described by Schmidt, Kirk, and Appleman (10). Their value of 0.2916 volt for the E.M.F. at 0° was used in the present work. We have determined the E.M.F. at 40° to be 0.2784 volt. In the latter instance the Nernst equation used in calculating the paH at 40° assumes the form:

$$\text{paH} = \frac{E_{40^\circ} - 0.2784}{0.06213}$$

while at 0°

$$\text{paH} = \frac{E_{0^\circ} - 0.2916}{0.05419}$$

It is perhaps desirable to state here that in carrying out measurements of hydrogen ion activity in amino acid solutions, poisoning of the electrode is frequently encountered. This is probably due to a slight decomposition of the amino acid. It is indicated by erratic voltage readings. In such cases platinum electrodes which have been recently replatinized must be employed and it is necessary when equilibrium is reached that the voltage readings remain constant.

For the purpose of estimating heat of solution a series of solubility estimations of the isoelectric amino acid at the given temperatures over the range of 0–47° was also carried out.

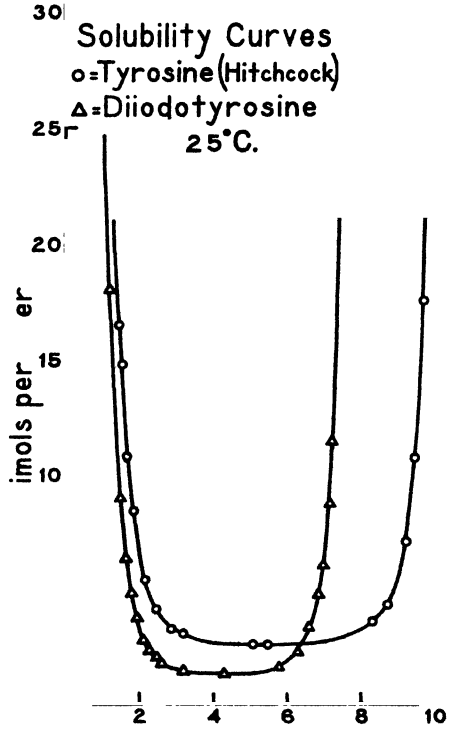

Fig. 1.

The solubility data for diiodotyrosine at 25° calculated in terms of millimols per liter together with Hitchcock's (7) data for tyrosine are represented graphically in Fig. 1. It is seen that the effect of the presence of iodine in the ring is to decrease the solubility of the iodine-containing compound over the isoelectric zone as well as to decrease the zone of minimum solubility. The latter effect is due to the increase in the values of the acid dissociation constants. In Table I we have summarized the data for the apparent dissociation constants of diiodotyrosine together with Hitchcock's constants for tyrosine. The data show that the value for $K'_b$ of diiodotyrosine at 25° is almost identical with the value for $K'_b$ given by Hitchcock for tyrosine, while the values for $K'_{a_1}$ and $K'_{a_2}$ respectively of diiodotyrosine are about a thousand times greater than the respective values for $K'_{a_1}$ and $K'_{a_2}$ for tyrosine. Mörner (11) has also shown that the introduction of halogens into the hydroxyphenyl ring markedly increases its acid properties.

TABLE I.*

| Substance. | Temperature. | $K'_{a_1}$ | $K'_{a_2}$ | $K'_b$ | pI |
|---|---|---|---|---|---|
| | °C. | | | | |
| Tyrosine (Hitchcock)......... | 25 | $7.8 \times 10^{-10}$ | $8.5 \times 10^{-11}$ | $1.57 \times 10^{-12}$ | 5.6 |
| Diiodotyrosine.. | 0 | $2.92 \times 10^{-7}$ | $4.94 \times 10^{-9}$ | $1.86 \times 10^{-13}$ | 4.37 |
| " . | 25 | $3.32 \times 10^{-7}$ | $1.51 \times 10^{-8}$ | $1.32 \times 10^{-12}$ | 4.29 |
| " .. | 40 | $3.51 \times 10^{-7}$ | $3.56 \times 10^{-8}$ | $3.72 \times 10^{-12}$ | 4.19 |
| $\Delta H'$ of diiodotyrosine.............. | | 810 calories. | 8790 calories. | 12770 calories. | |

* For the sake of consistency we have given the calculated rather than the observed values for $K_{a_1}$ and $K_b$. The observed values are given in Tables II and III.

In Fig. 2 the relations of the solubilities of diiodotyrosine in terms of mol fraction[1] with respect to hydrogen ion activity at various temperatures are shown. It is seen that the effect of decreasing the temperature is to decrease the numerical values of

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[1] We have made most of our calculations in terms of mol fraction as these units are consistent with thermodynamic reasoning while those in terms of mols per liter are not. One of the reasons for this selection is that the mol fraction of a solution is independent of the temperature while the concentration is not. Mol fraction may be defined as ($N_2 = \frac{n_2}{n_1 + n_2 + n_3}$ where $n_2$ is the number of mols of solute present, $n_1$ is the number of mols of solvent present, $n_3$ is the number of mols of other constituents, and $N_2$ is the mol fraction of the solute with which we are concerned.

the apparent acid and basic dissociation constants as well as to decrease the solubility of diiodotyrosine.

Since Hitchcock's (7) work indicated that the solubility of an amino acid at any given temperature and acidity is dependent upon the isoelectric solubility at that temperature we have carried out solubility estimations of diiodotyrosine at a number of temperatures in order to determine the exact relation between the solubility of this compound and the temperature. The data are shown graphically in Fig. 3. It has been found that the relationship between solubility and temperature can be expressed by the equation

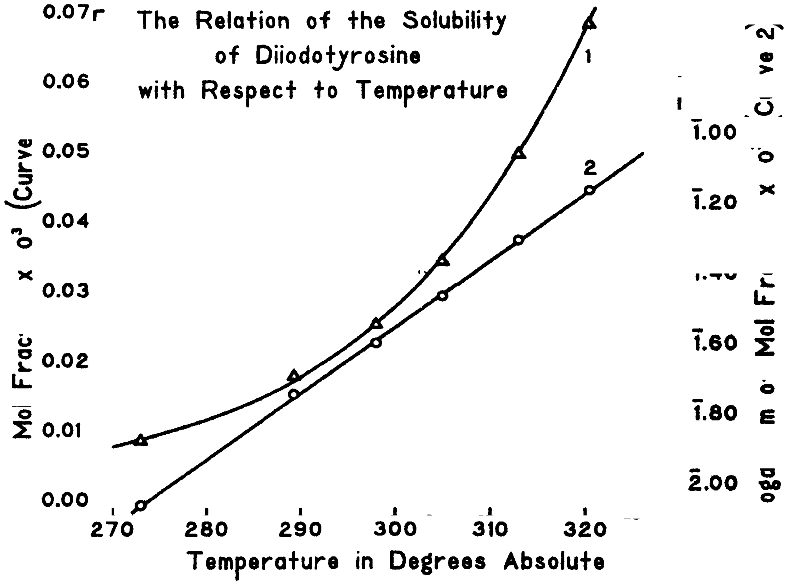

FIG. 2.

$$\log (N_2 \times 10^3) = 0.01923\ T - 7.329 \qquad (1)$$

where $T$ = absolute temperature and $N_2$ = mol fraction of the solute. On reducing the above equation from the logarithmic to the rational form we have

$$N_2 = 0.4688 \times 10^{(-10.0\ +\ 0.01923\ T)} \qquad (2)$$

Four of our experimental values fall directly on the plotted curve while the other two do not vary more than 3 per cent from it.

The thermodynamic relationship between $\Delta H$, the differential heat of solution; the mol fraction, $N_2$; the temperature, $T$; and the pressure, $P$, can be expressed as follows (12):

$$\left(\frac{\partial \ln N_2}{\partial T}\right)_P = \frac{\Delta H}{RT^2} \tag{3}$$

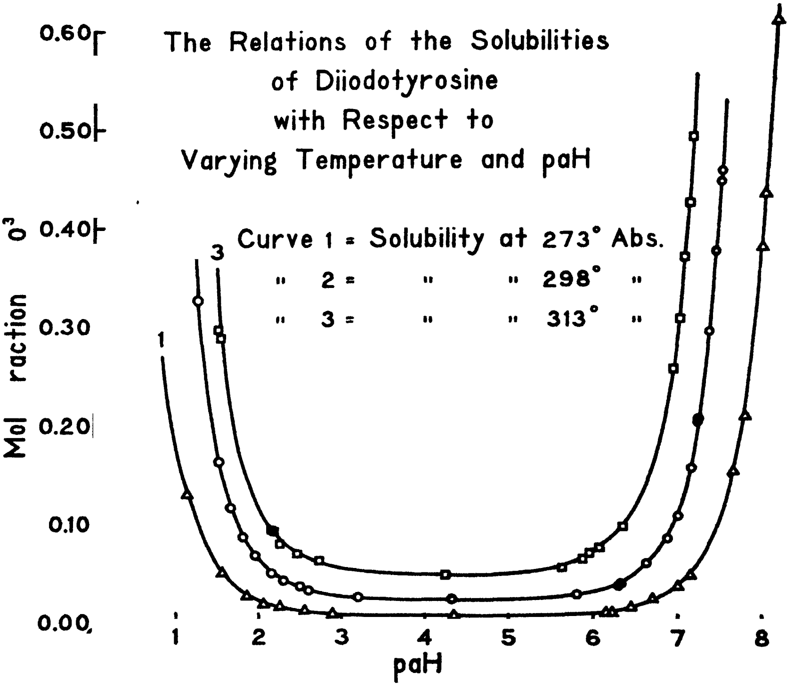

FIG. 3.

$\Delta H$ is also the total heat of solution of 1 mol of solute at infinite dilution. In dilute solutions such as we have used (0.002 M) the value for $\Delta H$ can be assumed to represent the total heat of solution.

Since $\log N_2 = 0.01923\,T - 10.329$ then $\ln N_2 = 0.04429\,T - 23.787$ or $\Delta H = 0.08807\,T^2$. At 298° absolute[2] the value of $\Delta H$ for diiodotyrosine is 7830 calories.

[2] More strictly 298.18.° See Birge, R. T., *Phys. Rev.* suppl., **1**, 1 (1929).

For the purpose of calculating the apparent heat of ionization of diiodotyrosine the equation of van't Hoff was used ((12) p. 298),

$$\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2}$$

or

$$-\Delta H = \frac{d(R \ln K)}{d\left(\frac{1}{T}\right)} \qquad (4)$$

where $T$ = the absolute temperature, $R$ = the gas constant in calories per degree, $K$ = true dissociation constant, and $\Delta H$ = the heat of ionization. Since the true dissociation constants of this amino acid are not known the assumption is made that the equation holds when the values for the apparent dissociation constants are used instead of the true dissociation constants. If $-R \ln K$ is plotted against $\frac{1}{T}$ the slope of the curve at any point is equal to the heat of ionization at that temperature. In most cases of dissociation this function is probably linear over small ranges of temperature. An exception to this is the dissociation of $NH_4OH \rightleftarrows \overset{+}{NH_4} + \overset{-}{OH}$ which over a range of 290° shows a distinct curve ((12) p. 314).

It was not found feasible to use temperatures much above 40° since diiodotyrosine decomposes to some extent in alkaline solutions at higher temperatures. The decomposition products tend to poison the platinum electrodes. Moreover, the uncertainty in the behavior of the hydrogen electrode at higher temperatures must also be considered.

The further assumption was made that $\Delta H'$, the apparent heat of ionization, is constant over the temperature range of 0–40°. We find that our data check this assumption with reasonable accuracy. The values for $-R \ln K'$ were plotted against the values for $\frac{1}{T}$, the best straight line was drawn between the points, and the equation which fits that line was determined.[3] In this manner we found for $K'_b$ that

$$-R \ln K'_b = \frac{12770}{T} + 11.52 \qquad (5)$$

---

[3] With the method of least squares the maximum deviation from the method herein used is not greater than 6 per cent.

and

$$-\log K'_b = \frac{2789}{T} + 2.52 \qquad (6)$$

$K'_b$ was calculated from Equation 6. In Table II the calculated values for $K'_b$ are shown in comparison with the observed values together with the percentage variation. Differentiating $-R \ln K'_b$ with respect to $\frac{1}{T}$ in Equation 5 the value for $\Delta H'$ is found to have the value of 12770 calories.

TABLE II.

*Calculated and Observed Values for the Apparent Basic Dissociation Constant of Diiodotyrosine.*

| $T$ absolute. | $K'_b$ calculated. | $K'_b$ observed. | Variation. |
|---|---|---|---|
| | | | *per cent* |
| 273 | $1.86 \times 10^{-13}$ | $1.73 \times 10^{-13}$ | +7 |
| 298 | $1.32 \times 10^{-12}$ | $1.49 \times 10^{-12}$ | −11 |
| 313 | $3.72 \times 10^{-12}$ | $3.56 \times 10^{-12}$ | +4 |

TABLE III.

*Calculated and Observed Values for the Apparent Acid Dissociation Constant ($K'_{a_1}$) of Diiodotyrosine.*

| $T$ absolute. | $K'_{a_1}$ calculated. | $K'_{a_1}$ observed. | Variation. |
|---|---|---|---|
| | | | *per cent* |
| 273 | $2.92 \times 10^{-7}$ | $3.00 \times 10^{-7}$ | −2.7 |
| 298 | $3.32 \times 10^{-7}$ | $3.10 \times 10^{-7}$ | +6.6 |
| 313 | $3.51 \times 10^{-7}$ | $3.78 \times 10^{-7}$ | −5.2 |

By a similar treatment we find for $K'_{a_1}$ that

$$-R \ln K'_{a_1} = \frac{806}{T} + 26.97 \qquad (7)$$

and

$$-\log K'_{a_1} = \frac{176}{T} + 5.89 \qquad (8)$$

$K'_{a_1}$ was calculated from Equation 8 and comparisons were made between the calculated and the observed values for $K'_{a_1}$. These are shown in Table III. Differentiating $-R \ln K'_{a_1}$ with respect to $\frac{1}{T}$ in Equation 7 we find that $\Delta H'$ has the value of 810 calories.

In attempting to calculate values for $K'_{a_2}$ certain difficulties were encountered. In Hitchcock's (7) equation

$$K'_{a_2} = \frac{(H^+)^2}{K'_{a_1}}\left[\frac{S}{S_0} - 1 - \frac{K'_a}{H^+}\right]$$

it will be noted that any errors in the value for $K'_{a_1}$ will be magnified in the values for $K'_{a_2}$. For example, if $K'_{a_1}$ was observed to have a smaller value than it should have, the value for the expression $\frac{(\mathrm{H}^+)^2}{K'_{a_1}}$ would be too large. This is also true for the expression $\left(\frac{S}{S_0} - 1 - \frac{K'_{a_1}}{\mathrm{H}^+}\right)$. It is evident that the total error is greater than is warranted by a small error in the value for $K'_{a_1}$.

TABLE IV.

*Calculated Values for the Second Apparent Acid Dissociation Constants ($K'_{a_2}$) of Diiodotyrosine (a) the Calculated Values for $K'_{a_1}$ and (b) the Observed Values for $K'_{a_1}$ Being Used.*

| $T$ absolute. | $K'_{a_2}$ using $K'_{a_1}$ calculated. | $K'_{a_2}$ using $K'_{a_1}$ observed. | Variation. |
|---|---|---|---|
| | | | *per cent* |
| 273 | $4.94 \times 10^{-9}$ | $3.89 \times 10^{-9}$ | 25 |
| 298 | $1.51 \times 10^{-8}$ | $1.96 \times 10^{-8}$ | 25 |
| 313 | $3.56 \times 10^{-8}$ | $3.16 \times 10^{-8}$ | 16 |

In order to evade this difficulty we have calculated $K'_{a_1}$ from Equation 8 and have used these values to calculate the values for $K'_{a_2}$. For comparison we have also calculated values for $K'_{a_2}$ using the observed values for $K'_{a_1}$. The data are summarized in Table IV.

We believe that the values obtained for $K'_{a_2}$, with calculated values for $K'_{a_1}$, are more desirable than the values which are obtained when the observed values for $K'_{a_1}$ are used since they are compensated by measurements which were made at other temperatures, while the values for $K'_{a_2}$ which are obtained when the observed values for $K'_{a_1}$ are used are subject to individual discrepancies. Using the more desirable values for $K'_{a_2}$ we find that

$$-R \ln K'_{a_2} = \frac{8791}{T} + 6.09 \tag{9}$$

and

$$-\log K'_{a_2} = \frac{1919}{T} + 1.33 \tag{10}$$

From Equation 10 values for $K'_{a_2}$ were calculated. Differentiating $-R \ln K'_{a_2}$ with respect to $\frac{1}{T}$ in Equation 9 we find that the value for $\Delta H'$ is 8790 calories.

Since $-\log K = \text{pK}$ it follows from Equations 6, 8, and 10 that pK is a function of the temperature, $T$. Knowing the temperature we then can calculate the value for the apparent dissociation constant at that temperature. Equation 2 is an expression showing the relation of the isoelectric solubility, $N_{2_0}$, to the temperature. Substituting the numerical values obtained in this equation in the Hitchcock (7) equation *viz*:[4]

$$N_2 = N_{2_0}\left[1 + \frac{K'_b H^+}{K_w} + \frac{K'_{a_1}}{H^+} + \frac{K'_{a_1} K'_{a_2}}{(H^+)^2}\right] \tag{11}$$

we obtain an expression for the solubility of diiodotyrosine at any temperature and acidity. Rewriting Equations 6, 8, and 10 respectively, we have

$$\text{pK}'_b = \frac{2789}{T} + 2.52 \text{ or } K'_b = \frac{1}{10^{\left(\frac{2789}{T} + 2.52\right)}}$$

$$\text{pK}'_{a_1} = \frac{176}{T} + 5.89 \text{ or } K'_{a_1} = \frac{1}{10^{\left(\frac{176}{T} + 5.89\right)}}$$

$$\text{pK}'_{a_2} = \frac{1919}{T} + 1.33 \text{ or } K'_{a_2} = \frac{1}{10^{\left(\frac{1919}{T} + 1.33\right)}}$$

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[4] We have utilized Hitchcock's equations in their general form but have used $K'_{a_1}$, $K'_{a_2}$, and $K'_b$ in place of $K_{a_1}$, $K_{a_2}$, and $K_b$. We have also used mol fraction where he used concentration.

Since $N_{2_0} = 0.4688 \times 10^{(-10 + 0.01923\,T)}$, substituting these values in Equation 11 we now have

$$N_2 = 0.4688 \times 10^{(-10 + 0.10923\,T)} \left[ 1 + \frac{(\mathrm{H}^+)}{K_w \times 10^{\left(\frac{2789}{T} + 2.52\right)}} + \frac{1}{(\mathrm{H}^+) \times 10^{\left(\frac{176}{T} + 5.89\right)}} + \frac{-}{(\mathrm{H}^+)^2 \times 10^{\left(\frac{2095}{T} + 7.22\right)}} \right]^*$$

Thus we have an expression for the solubility of diiodotyrosine at any temperature and acidity.

In order to check the accuracy of this expression we arbitrarily chose a point on the 298° curve (Fig. 3) at the observed paH of 7.011 and then calculated the solubility under these conditions. The observed solubility was 0.109 $\frac{\text{millimols}}{\text{mol solvent}}$ while the calculated solubility was 0.101 $\frac{\text{millimols}}{\text{mol solvent}}$. The deviation is about 8 per cent.

## SUMMARY.

1. From solubility estimations at various acidities we have determined the apparent acid and basic dissociation constants of diiodotyrosine at 0°, 25°, and 40°.
2. With these data the apparent heats of ionization of diiodotyrosine have been calculated.
3. The heat of solution of diiodotyrosine was determined by estimating the isoelectric solubility at a number of temperatures.

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[*] The value for $K_w$ at any temperature may be calculated from the expression $-RT \ln K_w = 29210 + 53\,T \ln T - 335.86\,T$.

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# THE ISOMERIZATION OF ERGOSTEROL WITH FULLERS' EARTH.

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Isoergosterols have been prepared by the catalytic action of several acids on ergosterol—hydrochloric acid (1, 2), hydrobromic acid (2), cinnamoyl chloride (2, 3), chloroacetic acid (2), sulfuric acid (4, 5). Other forms have been obtained chemically (6, 7). We have recently demonstrated (5) that the forms resulting from the acid catalysts are actually complex mixtures, difficult to separate.

Several years ago we showed (8, 9) that on contact with the so called acid clays, cholesterol gives cholesteryl ether, which upon prolonged treatment breaks down into a resinous mixture exhibiting antiricketic properties. Continuing this work with ergosterol, we have obtained an isoergosterol from the reaction mixture.

10 gm. of ergosterol, $[\alpha]_{5461}^{25} = -158°$ ($c = 1$ in $CHCl_3$), and 25 gm. of activated floridin (9) in 300 cc. of benzene were refluxed for 25 minutes. The catalyst rapidly underwent the color changes observed with cholesterol. The suspension was filtered, washed with hot benzene, and the combined filtrates were evaporated to dryness. The light yellow residue was comminuted with 400 cc. of alcohol, boiled for a few minutes, and filtered hot. An insoluble portion, about 15 per cent, was removed for future study.

The filtrate containing the isoergosterol was evaporated to dryness. The residue was crystallized twice from alcohol-benzene (2:1). The crystals were washed with a small quantity of ice-cold alcohol in each case. The crystals were then dissolved in boiling alcohol, and the solution was filtered to remove the last traces of alcohol-insoluble material. Recrystallization was continued with alcohol and with acetone, until a total of ten crystallizations had been made.

From each crystal crop and mother liquor samples were taken for polarimetric examination. They were dried in a high vacuum at 80° for 10 minutes, then dissolved in chloroform ($c = 1$). The first impure crystals gave $[\alpha]_{5461}^{25} = -19.2°$. With successive crystallizations the levorotation increased, rapidly at first, and then more gradually, the tenth crop showing $[\alpha]_{5461}^{25} = -37.3°$. From the rate of change in specific rotation, it seemed that an infinite number of crystallizations would have raised the levorotation to −40° or −41°. This is the limit also approached by the soergosterols prepared with HCl or HBr (5).

The mother liquors contained a dextrorotatory substance. The first liquor gave $[\alpha]_{5461}^{25} = +25.8°$. Successive liquors were less dextrorotatory, finally becoming levorotatory. Since the dextrorotatory liquors contained considerable pigment and resinous matter, no attempt was made to isolate the optically active substance.

The absorption spectrum of the crystal crops showed that each fraction was characterized by a single broad band with a maximum at 248 $m\mu$. This is the position of the maximum observed in the isoergosterols prepared with acid catalysts. The molecular extinction coefficients were somewhat low, $\epsilon = 13{,}000$, on account of a persistent contaminant of low extinction coefficient. The contaminant was particularly evident in the mother liquors. On treatment with hydrochloric acid the purified isomer behaved like the hydrochloric, hydrobromic, and cinnamic isomers (2, 5): it gave a new equilibrium of isomers indistinguishable from the original unpurified hydrochloric isomer mixture.

The purified floridin isoergosterol crystallized from acetone in well defined, colorless, acicular leaflets. It melted clear, without yellowing, at 142°. The clearing point was preceded by a sintering range of about 5°. This melting point is the same as that of the purified isoergosterols prepared with HCl or HBr (5).

Combustion gave the following results.

Theoretical for isoergosterol ($C_{27}H_{42}O$).......... C 84.74, H 11.07.
Found for 0.2 gm. sample of $x$ ................ " 84.5, " 10.9.

The molecular weight was determined from the melting point depression of a solution in *d*-camphor by the method of Rast (10). The found value was 397.4; the theoretical value was 382.3. Thus the found value was 4 per cent higher than the theoretical. By the same procedure the found molecular weight of purified ergosterol was 2 per cent less than the theoretical. In view of the high molecular weight, an error of 4 per cent is not extraordinary. All analytical data were obtained with samples dehydrated in a high vacuum at 80° for 10 minutes.

The alcoholic nature of this isomer was demonstrated by acetylation. 0.7414 gm. of isoergosterol and 2 cc. of acetic anhydride were heated for 10 minutes at 135–140°. The slightly yellow product, after evaporation to constant weight, weighed 0.8097 gm., which indicates that the sterol was 98.4 per cent acetylated. The acetate was crystallized twice from alcohol and once from acetone. The purified leaflets melted clear at about 149°, $[\alpha]_{5461}^{25} = -52.4°$ ($c = 1$ in $CHCl_3$). The saponification number was found to be 129.3 (phenolphthalein); the theoretical value for isoergosteryl acetate is 131.5. While it is unusual for a sterol ester to exhibit greater optical activity than its parent sterol, another such anomaly is found in the isoergosteryl chloroacetate described by Bills and Cox (2). In the present case, the amount of acetate was too small to permit an investigation of the sterol obtained by saponification.

Isoergosterol, like ergosterol, gives erratic iodine values, even with the Rosenmund-Kuhnhenn reagent. However, we found that at $-5°$ one is able to obtain with this reagent values indicative of three double bonds. The value found for isoergosterol was 203, and for ergosterol, 227; the theoretical value (three double bonds) is 199. While it is perhaps not justifiable to manipulate the conditions of the determination so as to obtain a desired value, this experiment at least indicates that isoergosterol is unsaturated to the same degree as ergosterol, and that it is slightly less reactive with a bromine solution (*cf.* MacLean (11), Reindel and Niederländer (12)).

The purified isoergosterol gave a modified Salkowski color test, the *sulfuric acid layer* becoming red. It gave also the Burchard-Liebermann test. With three drops of Rosenheim's aqueous trichloroacetic acid reagent 0.2 cc. of a 1 per cent solution gave pink → orchid → lavender → blue → green. However, with 18 drops of the acid added, it gave pink → lavendar → blue. According to Rosenheim (13), ergosterol itself gave an immediate red, changing to a clear blue without showing green. With ergosterol we obtained, employing 0.2 cc. of a 1 per cent solution and 3 to 6 drops of reagent, a red or pink which faded, changing to olive-green, and finally to a deep emerald-green without the blue. On the other hand, with 18 drops of reagent, there was developed a red, changing to blue. Therefore, it appears that concentration plays an important rôle in the color obtained. If the red coloration is specific for the $\Delta^{1,2}$ (or $\Delta^{1,13}$) ethenoid linkage, as suggested by Rosenheim, then it follows that isoergosterol and ergosterol are similar in this structure. Heilbron and Spring (4) have also obtained a positive Rosenheim test with their so called α- and β-isoergosterols, substances which may consist of several isomeric forms in admixture (5).

Like most other free sterols, the floridin isoergosterol was precipitated by digitonin. It was not antiricketic, and it did not become so to an appreciable degree on exposure to ultra-violet rays.

## SUMMARY.

1. Ergosterol in benzene solution was treated with fullers' earth. The reaction mixture contained an isoergosterol, an alcohol-insoluble substance, a dextrorotatory substance, and resinous decomposition products.

2. After ten crystallizations, the isoergosterol exhibited $[\alpha]_{5461}^{25} = -37.3°$, $\epsilon = 13{,}000$, m.p. = 142°. The acetyl derivative gave $[\alpha]_{5461}^{25} = -52.4°$, m.p. = 149°.

3. Determinations of the iodine value at −5° indicated that the isoergosterol had the same number of double bonds as ergosterol. Rosenheim's color reaction indicated that one of the bonds was the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage of ergosterol.

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# AN IMPROVED METHOD OF MEASURING GLASS ELECTRODE POTENTIALS.

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Since Haber and Klemensiewicz (1) in 1909 first suggested that the change in potential between a thin glass interface and a solution would serve as an indicator in following the variation in pH, several noteworthy investigations have appeared in the literature. Among the most valuable are those of Kerridge, Hughes, and MacInnes and Dole, for through their efforts the glass electrode now affords a valuable method for the determination of hydrogen ion activities. The admirable research carried out by MacInnes and Dole (2) on the behavior of glass electrodes of different compositions resulted in the finding of a glass electrode possessing the suitable characteristics of complete reversibility, low asymmetry potential, and low resistance. Their investigation also indicated that this electrode was as accurate as the hydrogen electrode in a pH range not exceeding 9.5, provided the concentration of salt in the solution was 0.1 N or less.

Wide-spread use of the glass electrode is, however, limited by the difficulties encountered in measuring the E.M.F. of a high resistance system. Various types of electrometer set-ups have been employed, but they are expensive and are difficult to operate in a routine fashion. Numerous vacuum tube potentiometers have also been described in the literature, but the fault common to all is that the E.M.F. observed is not the true E.M.F. of the cell, but a value represented by the following equation.

$$E_d = E_t \pm i_g \cdot R_g$$

where

$E_d$ = observed potential
$E_t$ = true potential
$i_g$ = grid current
$R_g$ = cell resistance

Obviously, any change in either grid current or in cell resistance during the course of a series of measurements would introduce an error. Therefore, the determination of the *cell constant* would not obviate this difficulty.

One of us (Fosbinder) has described elsewhere (3) the theory and construction of a single tube—vacuum tube potentiometer for determining the true E.M.F. of a high resistance cell. The chief advantages of this type of potentiometer are inexpensiveness, stability, ease of control, and high sensitivity. Since only one tube is used, its characteristics need not be determined; the fact that glass electrodes of rather high resistance may be employed without loss of sensitivity permits the use of rather sturdy electrodes, an obvious advantage. Considerable difficulty has been encountered heretofore not only in the measurement of the E.M.F. but likewise in the design of a cell whose temperature may be thermostatically controlled, and of a form permitting the use of small volumes of fluid (0.05 to 0.2 cc.) with accuracy and convenience. With the use of an improved form of the vacuum tube potentiometer and a cell of new design, to be described later, the p$\alpha$H values of phosphate buffer solutions have been determined at 38°, and compared with the p$\alpha$H values of the same buffer mixtures determined with the Simms type of hydrogen electrode in order to test the reversibility and accuracy of the glass electrodes.

The assembled apparatus is shown in Fig. 1, in which *A* is the thermostat, *B* the vacuum tube potentiometer, *C* a laboratory H ion potentiometer, and *D* a galvanometer. The vacuum tube potentiometer proper contains the cell, vacuum tube, control panel, high resistance switches, and potentiometer leads. The instrument panel is of Bakelite, the box is constructed of brass and is grounded in order to eliminate electrostatic and body capacity effects. Since the switches $S_1$, $S_2$, and $S_3$, are connected with the grid, they must be provided with suitable insulation so that the leakage current from the switches is at least as small as that from grid to ground, also, the switches must be adequately shielded. The details of construction of the switch are shown in Fig. 2. The contacts are made of gold in order to avoid the effect of contact potentials. All leads, except those of the potentiometer and grid circuit, are connected to tip jacks, which are mounted on a Bakelite panel on the lower inside right end of the box. Short-circuiting of the jacks by the brass is avoided by drilling holes slightly larger than the jack shaft. The short potentiometer leads are brought out in copper tubing and are highly insulated with sulfur. The tube is further protected by a standard screen grid tube shield. The component parts of the control panel, reading from left to right and beginning with the top row, are given below, together with the respective symbols designated in the wiring diagram (see Fig. 3). $R_3$ represents a 25 ohm rheostat; $R_6$, 2000 ohm potentiometer; $MA$, milliammeter; $R_1$, 10,000 ohm rheostat; $R_2$, 5000 ohm rheostat; $R_5$, 200 ohm rheostat; $R_4$, 5 ohm rheostat, $S_4$, type K switch; $R_3$, 100 ohm rheostat; $S_1$, $S_2$, $S_3$, three insulated Bakelite switches for batteries. The remaining details of the apparatus may be found in the article previously mentioned (3).

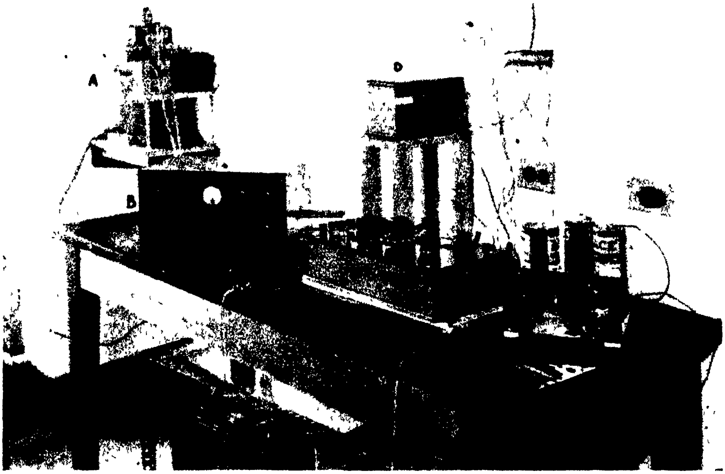

FIG. 1. Photograph of apparatus for measurement of glass electrode potentials, showing thermostat, vacuum tube potentiometer, galvanometer, and H ion potentiometer. The set-up at the right is for the purpose of measuring the resistance of glass electrodes.

Since the pH cell is a source of high resistance, and is in series with the grid, it must be provided with high resistance insulation. To this end the cell is supported by means of two clamps secured to Bakelite rods which are firmly embedded in a base of the same material. By the use of a hinge, the base of the cell support is fastened to the rear side of the box, thus permitting the support to swing through an arc. This arrangement allows easy accessibility to the cell at all times for purposes of cleaning and filling.

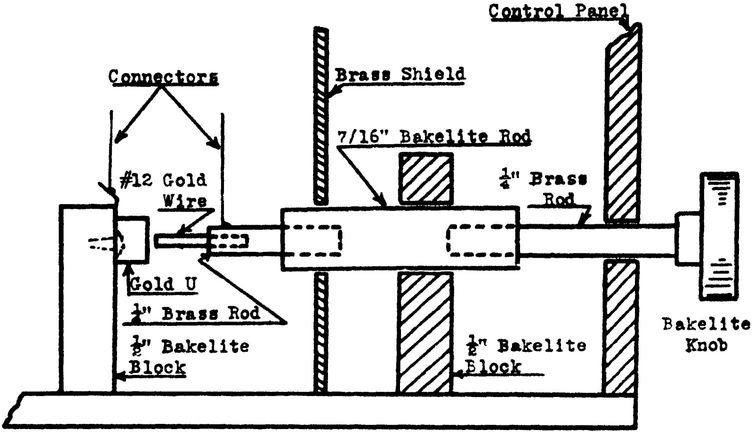

Fig. 2. Cross-section diagram of shielded high insulation switch employed in the grid circuit.

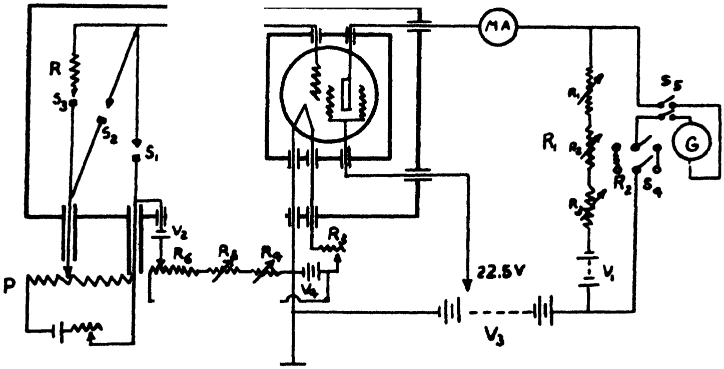

Fig. 3. Diagram of working circuit used to determine the E.M.F. of high resistance cell. See text for explanation of the symbols.

Leads from the cell are directly connected with two brass contacts which are fastened on the rear of the base. A similar set of brass strips, mounted on a piece of Bakelite, is connected in the grid circuit. When the cell support is swung into position, the contacts engage, thereby inserting the cell in the circuit. The cell and its support are shown in Fig. 4 in which *a* is a jacketed saturated calomel electrode, *b* is a saturated KCl bridge and reservoir, *c* a jacketed fluid cell, *d* a rack and pinion arrangement for adjusting

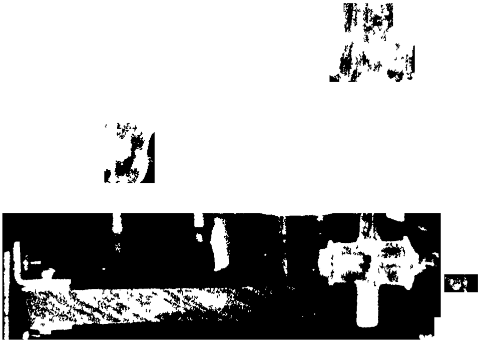

FIG. 4. Photograph of cell used in the determination of H ion activities. The liquid whose H ion activity is to be measured is placed either in the capillary, *f*, or in the surrounding cup. The symbols are explained in the text.

the height of the glass electrode, which is held in position in clamp *e*, above the end of the capillary, *f*, which is the container for the fluid.

A sectional diagram of the cell, shown in Fig. 5, reveals the details of its construction. The apparatus with the exception of the calomel electrode connector, *e*, is made of Pyrex glass.

Soft glass must be employed in the connector in order to permit the fusing in of a platinum wire. The ground glass joint, *d*, of the calomel cell, *a*, eliminates the often troublesome rubber stopper. The reservoir, *b*, is a container for supplying the bridge, *g*, with saturated KCl. The design of the fluid cell, *c*, permits the use of either large or small volumes of solution as the space surrounding the exposed portion of the capillary, *f*, may be employed. To provide an inlet and outlet for the circulating bath liquid tubes, *h*, are attached to the rear side of the cell jackets. The rubber tubes which lead to and from the cell jackets are joined to glass tubes so mounted in Bakelite rods that they are free to move when the cell support is moved. The connections to the thermostat are made from the glass tubing on the outside of the box.

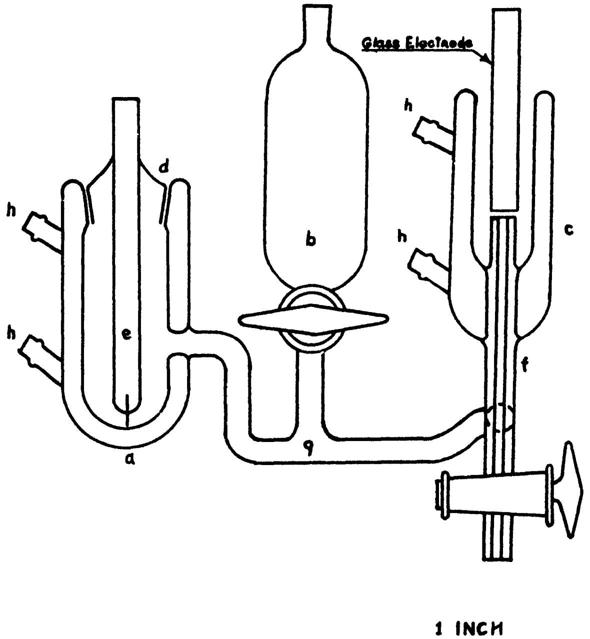

FIG. 5. Full scale cross-section diagram of the pH cell shown in Fig. 4.

The use of a water-filled thermostat for controlling the temperature of the cell may result in considerable difficulty, due to the presence of very small leakage currents which cause the plate current to be reduced. This difficulty may be eliminated either by the use of an oil thermostat or by taking all leakage currents to ground. It is also necessary to use an induction motor in connection with the thermostat in order to avoid effects from sparking which are encountered with the brush type motor. In place of the usual type of relay it is advisable to use an ordinary telegraph sounder, with a mercoid switch mounted on the sounder bar.

The operation of the vacuum tube potentiometer has been described before (3) and hence will not be given here. To determine the p$\alpha$H of a sample of fluid the capillary is filled with KCl to the top by opening the stop-cock of the reservoir. The sample, approximately 0.1 cc., is introduced from a rubber-tipped 1 cc. tuberculin syringe by tightly holding the tip of the syringe against the end of the capillary and opening stop-cock *g*. When the requisite amount has entered the capillary, the stop-cock is closed and the syringe removed. The glass electrode, similar to the type employed by MacInnes and Dole, containing 0.1 N hydrochloric acid, and in which is inserted a silver chloride-silver electrode, is placed in the clamp, and the electrode lowered by means of the rack and pinion until contact is made with the fluid on the tip of the capillary. A new junction at the glass surface is made by simply opening the reservoir stop-cock, thus forcing a drop of the fluid over the edge of the capillary. Before introducing the electrode into the cell, it is carefully rinsed on the outside with the solution whose pH is to be determined. Any drops adhering to the glass membrane may be removed by carefully touching the surface with the edge of a piece of filter paper. To prepare the cell for a fresh sample, the glass electrode is removed and the capillary rinsed several times with KCl solution by manipulation of the two stop-cocks.

The 0.1 N hydrochloric acid and the silver-silver chloride electrodes employed in the determinations were checked by determining, at 25°, the E.M.F. of the following cell.

$$H_2 \mid 0.1\ \text{N}\ HCl,\ AgCl \mid Ag$$

The M/15 $Na_2HPO_4$ and M/15 $KH_2PO_4$ solutions were prepared with freshly distilled water and were kept at 7° except during the withdrawal of the portions for the buffer mixtures. The glass used in making the electrodes was of similar composition to that employed by MacInnes and Dole, and was purchased from the Corning Glass Works.

The p$\alpha$H values of the phosphate buffer mixtures were determined by means of the hydrogen electrode, with the following type of cell.

$$\text{Hg} \mid \text{Hg}_2\text{Cl}_2, \text{ saturated KCl} \parallel \text{buffer} \mid \text{H}_2$$

By means of the cell combination given below the p$\alpha$H values of the same buffer solutions were determined with the glass electrode.

$$\text{Hg} \mid \text{Hg}_2\text{Cl}_2, \text{ saturated KCl} \parallel \text{buffer} \mid \text{glass} \mid 0.1 \text{ N HCl, AgCl} \mid \text{Ag}$$

Before each series of buffer determinations, the hydrogen electrode was carefully checked with 0.1 N HCl. All measurements reported in this paper were made at a temperature of 38° and the p$\alpha$H values are referred to the following standard of reference: p$\alpha$H of 0.1 N HCl at 38° = 1.08.

In any series of glass electrode determinations, a reference value was selected and the other p$\alpha$H values calculated by means of the equation:

$$\Delta E = 0.0617 \, \Delta \text{p}\alpha\text{H}$$

Table I contains the results of a series of measurements carried out with phosphate buffers in which the hydrogen and glass electrodes were used.

An approximate computation of the asymmetry potentials of the glass electrodes employed may be carried out with the aid of the following data. The E.M.F. of the cell

$$\text{H}_2 \mid 0.1 \text{ N HCl, AgCl} \mid \text{Ag}$$

at 38° is −0.3491 volt. If the potential of the saturated calomel cell is assumed to be 0.236 volt at the same temperature, the theoretical potentials of the glass electrode for various pH values may be calculated by means of the equation:

$$E = -0.3491 + 0.236 + 0.0617 \text{ p}\alpha\text{H}$$

The difference between the calculated and observed potentials yields the asymmetry potential of the glass electrode.

The resistances of the glass electrodes employed varied from 20 to 75 megohms. The high resistances of the electrodes are explained by the fact that electrodes with rather thick membranes were purposely constructed in order to reduce breakage to a minimum.

TABLE I.

*Values of Phosphate Buffers with Hydrogen and Glass Electrodes.*

| Buffer ratio, $Na_2HPO_4$: $KH_2PO_4$. | Date. | Glass electrode No. | E.M.F. | | pαH | | |
|---|---|---|---|---|---|---|---|
| | | | Hydrogen (uncorrected). | Glass. | Hydrogen. | Glass. | Deviation. |
| 3:7 | Apr. 7 | 7a | 0.6377 | 0.2842 | 6.39 | 6.39 | 0.00 |
| | " 10 | 3a | 0.6357 | 0.2832 | 6.36 | 6.39 | +0.03 |
| | " 18 | 10a | | 0.3382 | 6.38 | 6.37 | −0.01 |
| | " 22 | 9a | 0.6381 | 0.3016 | 6.38 | 6.38 | 0.00 |
| 4:6 | " 4 | 7a | 0.6480 | 0.2979 | 6.56 | 6.57 | +0.01 |
| | " 10 | 3a | 0.6492 | 0.2948 | 6.58 | 6.58 | 0.00 |
| | " 17 | 9a | 0.6496 | 0.3282 | 6.58 | 6.59 | +0.01 |
| | " 18 | 10a | | 0.3520 | 6.57 | 6.59 | +0.02 |
| 5:5* | " 2 | 6a | 0.6561 | 0.3061 | 6.69 | 6.65 | −0.04 |
| 5:5 | " 4 | 7a | 0.6588 | 0.3086 | 6.74 | 6.74 | 0 00 |
| | " 10 | 3a | 0.6594 | 0.3039 | 6.76 | 6.77 | +0.01 |
| | " 17 | 9a | 0.6600 | 0.3380 | 6.75 | 6.75 | 0.00 |
| | " 18 | 10a | | 0.3621 | 6.75 | 6.75 | 0 00 |
| 6:4 | " 7 | 7a | 0.6703 | 0.3165 | 6.92 | 6.91 | −0.01 |
| | " 17 | 9a | 0.6705 | 0.3483 | 6.92 | 6.92 | 0.00 |
| | " 18 | 10a | | 0.3720 | 6.92 | 6.92 | 0.00 |
| 7:3* | " 2 | 6a | 0.6770 | 0.3297 | 7.03 | 7.03 | 0.00 |
| 7:3 | " 4 | 7a | 0.6810 | 0.3305 | 7.10 | 7.10 | 0.00 |
| | " 21 | 9a | 0.6822 | 0.3459 | 7.10 | 7.10 | 0.00 |
| 8:2 | " 4 | 7a | 0.6953 | 0.3439 | 7.33 | 7.32 | −0.01 |
| | " 21 | 9a | 0.6963 | 0.3601 | 7.32 | 7.33 | +0.01 |
| | " 23 | 7a | 0.6965 | 0.3411 | 7.34 | 7.34 | 0.00 |
| | " 25 | 7a | | 0.3409 | 7.34 | 7.34 | 0.00 |
| 9:1 | " 7 | 7a | 0.7157 | 0.3624 | 7.66 | 7.66 | 0.00 |
| | " 21 | 9a | 0.7182 | 0.3790 | 7.68 | 7.64 | −0.04 |
| | " 23 | 7a | 0.7188 | 0.3612 | 7.70 | 7.67 | −0.03 |
| 9:1† | " 24 | 7a | 0.7226 | 0.3669 | 7.77 | 7.76 | −0.01 |
| 9½:½ | " 21 | 9a | 0.7374 | 0.3982 | 7.99 | 7.95 | −0.04 |

* 0.1 M.

† 0.03 M.

In general, if the electrodes are soaked in dilute hydrochloric acid or water for about 72 hours before use they come to equilibrium rapidly. On the other hand, if they do not receive this treatment, several hours are required before a steady potential is obtained.

## DISCUSSION.

A review of the data presented reveals two outstanding facts, one of which is that the glass electrode is entirely reversible in the pH range studied, the other, that it is apparently as accurate as the hydrogen electrode. In the most alkaline mixture used the buffer was diluted in order to preclude any possible salt effect. In agreement with MacInnes and Dole we find that it is quite advisable to select an electrode having a low asymmetry potential if accurate results are to be obtained.

It is quite obvious that the glass electrode has applications in many fields. As an illustration, we may state that we have successfully determined the pH values of nitrocellulose solutions in acetone.

## SUMMARY.

An improved form of vacuum tube potentiometer has been described, which, in connection with a constant temperature cell of new design, permits the determination of the p$\alpha$H of as little as 0.05 cc. of fluid with accuracy.

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# THE CYSTINE DEFICIENCY OF THE PROTEINS OF GARDEN PEAS AND OF POTATOES.

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In a previous publication (1) it was shown that the proteins of cooked (canned) green garden peas are deficient in cystine, since the growth of rats receiving a ration limited in nutritive value only in respect to its content of pea protein was stimulated by small additions of cystine over that of control rats receiving the same amount of unsupplemented basal ration. It was also shown by the same method, though less certainly because of the smaller number of animals used, that the nitrogenous substances of potatoes (peeled) are deficient in cystine for animal growth. Since the cystine deficiency of the garden peas may have been due to the cooking to which they had been subjected, the experiment was repeated on fresh green garden peas. The potato experiment was also repeated with a larger number of rats.

The paired feeding method was used in this as well as in the preceding experiments. In this method each test animal has its own control, of the same sex, approximately of the same initial weight, and generally of the same litter, which throughout the feeding period receives the same amount of food and of the same composition except for the inclusion of a small amount of cystine and the slight adjustment of other constituents necessary to give rations of equal nitrogen content.

The garden peas were obtained from the Horticulture Department of the University of Illinois. They were shelled, passed through a hand mill, dried at a low temperature, and reground.

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* On leave of absence from the Institute of Animal Nutrition, Pennsylvania State College, State College.

In their final form they contained 5.21 per cent of water, 3.73 per cent of nitrogen, 4.49 per cent of crude fiber, 2.73 per cent of ether-soluble material, and 3.66 per cent ash. The potatoes (Ohio Red variety) were boiled, peeled, riced, dried at a low temperature, and ground. They were analyzed for nitrogen only, two preparations containing 1.65 and 1.74 per cent.

The average formulas used in making up the rations as well as their nitrogen and crude protein contents are given in Table I. After a most thorough mixing of the finely ground constituents the rations were dried in a low temperature oven. The protein content of each ration, approximating 8 to 9 per cent, was low enough so that it was the limiting factor in its growth-promoting value. Besides the experimental rations, the rats were given daily small amounts of vitamin concentrates, cod liver oil, tikitiki, and occasionally dried yeast. These addenda were varied according to the size of the rats and were increased occasionally to improve a failing appetite, but at all times pair mates received the same amounts of these materials.

TABLE I.

*Percentage Composition of Rations.*

| Constituents. | Peas. | Peas and cystine. | Potato. | Potato and cystine. |
|---|---|---|---|---|
| Peas, garden, fresh | 34.32 | 33.29 | | |
| Potato | | | 75.48 | 73.23 |
| Cystine | | 0.24 | | 0.24 |
| Salts, Osborne and Mendel* | 4 00 | 4.00 | 4.00 | 4 00 |
| Butter fat | 10.00 | 10.00 | 10.00 | 10.00 |
| Sucrose | 10.00 | 10.00 | 8.69 | 8.69 |
| Sodium chloride | 1.00 | 1.00 | 1.00 | 1.00 |
| Starch | 40 68 | 41.47 | 0.83 | 2 84 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 |
| Nitrogen | 1.393 | 1.396 | 1.266 | 1.290 |
| Crude protein | 8.706 | 8.725 | 7.912 | 8.062 |

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

Nine pairs of rats were used in the experiment with garden peas, eight pairs of young rats less than 70 gm. in weight, and one pair of half grown rats. The feeding period lasted 9 weeks for the young rats and 61 days for the older pair. The initial and final weights of the rats were determined by averaging in each case the weights on 3 consecutive days. Weights were also taken at the end of each experimental week. The results of this test are summarized in Table II.

In each of the nine pairs of rats the rat fed cystine gained the more, a result that would have been obtained by chance only once in 512 trials. The average difference in total gains between pair mates is 12.8 gm., and the standard deviation of these differences is 6.98. The mean is thus 1.8 times the standard deviation and according to "Student's" (2) tables for assessing the significance of the means of small samples, the probability is less than 0.0005 that a mean of nine variates will, by chance only, exceed the standard deviation of the sample by as much or more than this. The experiment clearly demonstrates, therefore, that the one differ-

TABLE II.

*Value of Cystine As Supplement to Proteins of Fresh Green Garden Pea.*

All weights are given in gm.

| Pair No. | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | | 8 | | 9 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control. | Cystine. | Control. | Cystine. | Control. | Cystine. | Control. | Cystine. | Control. | Cystine. | Control | Cystine. | Control. | Cystine. | Control. | Cystine. | Control. | Cystine. |
| Initial weight | 62 | 64 | 66 | 67 | 66 | 66 | 68 | 69 | 69 | 67 | 55 | 55 | 50 | 47 | 54 | 54 | 138 | 134 |
| Final " | 93 | 98 | 99 | 109 | 83 | 95 | 88 | 105 | 109 | 127 | 96 | 113 | 89 | 100 | 92 | 109 | 187 | 210 |
| Gain | 31 | 34 | 33 | 42 | 17 | 29 | 20 | 36 | 40 | 60 | 41 | 58 | 49 | 53 | 38 | 55 | 49 | 76 |
| Total food | 324 | 324 | 340 | 340 | 276 | 280 | 324 | 323 | 377 | 376 | 311 | 309 | 312 | 308 | 345 | 345 | 546 | 543 |
| Days fed | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 61 | 61 |

ence between test and control rations, namely the added cystine, was instrumental in securing more rapid gains. Since the only known way in which cystine could exert this favorable effect in a ration deficient only in protein is by improving the value of the protein for growth, it may be concluded that fresh garden peas contain a protein mixture which is biologically deficient in this amino acid.

It is interesting to note how consistently the effect of cystine was brought out by this feeding method involving the equalization of the consumption of food between each experimental animal and its control. A comparison of the weekly gains of pair mates will serve this purpose. In Table III these comparisons are made for each pair for each week of the experiment. In Table III a plus sign indicates a greater gain by the rat fed cystine, a minus sign a greater gain by the control rat, and a plus-minus sign an equal gain by the pair mates.

TABLE III.

*Comparison of Weekly Gains Made by Pair Mates in Experiment on Fresh Garden Peas.*

| Wk. | Pair 1. | Pair 2. | Pair 3. | Pair 4. | Pair 5. | Pair 6. | Pair 7. | Pair 8. | Pair 9. | Total per wk. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | + | − | ± |
| 1 | + | − | − | + | ± | + | + | + | + | 6 | 2 | 1 |
| 2 | + | + | + | + | + | + | − | + | + | 8 | 1 | 0 |
| 3 | − | − | + | + | + | − | + | ± | + | 5 | 3 | 1 |
| 4 | ± | − | − | + | + | + | + | + | + | 6 | 2 | 1 |
| 5 | ± | + | ± | + | + | − | + | + | ± | 5 | 1 | 3 |
| 6 | + | + | ± | + | + | + | + | + | ± | 7 | 0 | 2 |
| 7 | − | + | + | + | + | + | + | + | + | 8 | 1 | 0 |
| 8 | + | + | + | − | + | + | + | + | + | 8 | 1 | 0 |
| 9 | − | − | − | + | + | ± | + | − | + | 4 | 4 | 1 |
| Totals. + | 4 | 5 | 4 | 8 | 8 | 6 | 8 | 7 | 7 | 57 | | |
| − | 3 | 4 | 3 | 1 | 0 | 2 | 1 | 1 | 0 | | 15 | |
| ± | 2 | 0 | 2 | 0 | 1 | 1 | 0 | 1 | 2 | | | 9 |

+ indicates a greater gain by the rat in the pair fed cystine, − a greater gain by the control rat, and ± equal gains by pair mates.

In every week of the experiment except the last the gains by the rats receiving the ration supplemented with cystine exceeded those of their pair mates in the majority of the pairs, and for the entire experiment there were 57 comparisons favoring the cystine rats, nine comparisons favoring neither rat, and only fifteen comparisons favoring the control rat.

The experiment on potato proteins involved eight pairs of rats. At the end of 2 weeks one pair was removed because of poor appetite and general malnutrition and another pair was substituted for it. The feeding period lasted 9 weeks for the original seven pairs, and seven weeks for the substituted pair. The results of this test are summarized in Table IV.

TABLE IV.

*Value of Cystine As Supplement to Proteins of Potato.*

All weights are given in gm.

| Pair No. | 1 | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|
| | Control. | Cystine. | Control. | Cystine | Control. | Cystine. | Control. | Cystine. |
| Initial weight | 66 | 65 | 54 | 53 | 71 | 69 | 67 | 66 |
| Final weight* | 102 | 115 | 94 | 121 | 96 | 120 | 109 | 123 |
| Gain | 36 | 50 | 40 | 68 | 25 | 51 | 42 | 57 |
| Total food | 316 | 319 | 395 | 395 | 382 | 385 | 383 | 386 |
| Days fed | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 |

| Pair No. | 5 | | 6 | | 7 | | 8 | |
|---|---|---|---|---|---|---|---|---|
| | Control. | Cystine. | Control. | Cystine. | Control. | Cystine. | Control. | Cystine. |
| Initial weight | 66 | 68 | 56 | 54 | 54 | 54 | 86 | 82 |
| Final weight.* | 124 | 145 | 102 | 115 | 80 | 109 | 125 | 129 |
| Gain | 58 | 77 | 46 | 61 | 26 | 55 | 39 | 47 |
| Total food | 444 | 444 | 357 | 355 | 307 | 311 | 336 | 342 |
| Days fed | 63 | 63 | 63 | 63 | 63 | 63 | 49 | 49 |

* The final weights of rats in this experiment are the weights at the end of the last day of feeding, rather than averages of 3 consecutive days, as in all other initial and final weights reported in this paper.

In each of the eight pairs, the rat receiving the added cystine made the more rapid gain, a result that would have occurred by chance only once in 256 trials. The average difference between the total gains of pair mates is 19.25 gm. and the standard deviation of the eight differences is 7.14 gm. The mean is thus almost 2.7 times the standard deviation and the probability of its occurrence by chance is, according to "Student's" table, extremely small.

The comparisons between the weekly gains of pair mates are summarized in Table V. Starting with the 4th week, the evidence consistently and strongly favors the pair mates receiving the cystine supplement. In the entire experiment, 50 comparisons favor the cystine rat, five favor neither pair mate, and only fifteen favor the control rat.

TABLE V.

*Comparison of Weekly Gains Made by Pair Mates in Experiment on Potato.*

| Wk. | | Pair 1. | Pair 2. | Pair 3. | Pair 4. | Pair 5. | Pair 6. | Pair 7. | Pair 8. | Total per wk. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | + | − | ± |
| 1 | | + | ± | + | − | ± | − | + | | 3 | 2 | 2 |
| 2 | | + | − | + | − | − | − | + | | 3 | 4 | 0 |
| 3 | | ± | ± | + | + | + | − | − | + | 4 | 2 | 2 |
| 4 | | + | + | + | + | + | + | + | − | 7 | 1 | 0 |
| 5 | | + | + | + | + | + | + | − | + | 7 | 1 | 0 |
| 6 | | + | + | − | + | + | + | + | − | 6 | 2 | 0 |
| 7 | | − | + | + | + | + | + | + | + | 7 | 1 | 0 |
| 8 | | − | + | + | + | + | + | + | − | 6 | 2 | 0 |
| 9 | | + | + | + | + | + | + | ± | + | 7 | 0 | 1 |
| Totals. | + | 6 | 6 | 8 | 7 | 7 | 6 | 6 | 4 | 50 | | |
| | − | 2 | 1 | 1 | 2 | 1 | 3 | 2 | 3 | | 15 | |
| | ± | 1 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | | | 5 |

+ indicates that the rat fed cystine gains the faster, − that the control rat gains the faster, and ± that pair mates gain the same.

All of this evidence is quite decisive in showing that cystine supplements the potato proteins, by which is meant the nitrogenous compounds of potato capable of serving in protein synthesis in the body.

An integral part of these paired feeding experiments as they are being standardized in this laboratory is the systematic recording of all refusals of feed from day to day, necessitating a restriction in the daily food portion of both rats of a pair. The feeding is designed to keep one rat in each pair practically at full feed, so that the refusals of food mark the rats which are taking their food with less avidity and hence are setting the pace for their pair mates. If the belief quite commonly held that the more complete of two rations will be consumed in the greater amount were true, one would expect in these cystine experiments that whenever cystine improves the growth-promoting value of a ration, the ration supplemented with cystine would be consumed more readily than the ration containing no added cystine. Our records of food refusals do not bear this out, however.

In the potato experiment just considered there were thirty-six refusals of food by control rats to only nine by the rats fed cystine, but in the garden pea experiment, there were only twenty-one refusals of feed by the control rats to forty-seven by the rats fed cystine. Going back to the cystine experiments by the paired feeding method previously reported (1), during which such records were first made, we find that, in the trial with canned garden peas, there were 59 refusals of feed among the control rats to 80 among the cystine rats, while in the experiments with milk there were forty-eight refusals of feed among the controls to forty-two among the rats fed cystine. In both of these experiments the evidence was conclusive that the unsupplemented rations were deficient in cystine.

The paired feeding experiments do not support the assumption that the more complete of two rations will always, or even generally, be consumed in the greater amount.

## CONCLUSIONS.

In paired feeding experiments involving nine and eight pairs of rats, respectively, it has been shown that the growth-promoting value of the proteins (N × 6.25) of fresh green garden peas and of boiled and peeled potatoes is enhanced by the addition of cystine. Hence, these protein mixtures are deficient in this amino acid.

In a number of experiments in which the growth-promoting value of a ration was increased by the inclusion of cystine within it, we have not been able to show that the supplemented ration was consumed with any more avidity than the unsupplemented.

These experiments afford no support for the assumption that the more complete of two rations will always, or even generally, be consumed in the greater amount.

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# THE RELATION BETWEEN CYSTINE DEFICIENCY IN THE DIET AND GROWTH OF HAIR IN THE WHITE RAT.

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The paired feeding experiments described in the preceding paper presented an opportunity to obtain some direct information on a question that has been recently revived by Lightbody and Lewis (1); *i.e.*, the relation between the cystine content of the diet and hair growth. Lightbody and Lewis concluded from their experiments on different groups of white rats receiving rations containing 16.70 per cent of whole milk powder, with graded additions of casein from 0 to 16 per cent, that, "The amount of hair produced, like the general somatic development, was related to the protein (and cystine) content of the diet, but under the experimental conditions of the present study, the demands for protein (and cystine) for the growth of the hair appeared to be secondary in importance to the demands for growth of the body with its more essential tissues." However, these experiments were not planned so as to afford any direct information on the relation between cystine and hair growth, since in no case was cystine a sole variable between any two diets. In fact, the implication that cystine was specifically involved in the greater hair growth obtained on the rations containing the higher concentrations of casein, seems quite inconsistent with the known deficiency of casein in this amino acid.

In the experiments described in the preceding paper, seventeen pairs of rats were fed upon a low protein diet (8 to 9 per cent). The protein mixture in each of the two diets used was demon-

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* On leave of absence from the Institute of Animal Nutrition, Pennsylvania State College, State College.

strably deficient in cystine. One rat in each of the seventeen pairs received a small cystine supplement, but the intake of food was the same for all pair mates. In each pair the rat receiving added cystine grew faster than its control mate on the same amount of food, a specific result of the cystine addendum.

TABLE I.

*Growth of Hair on Garden Pea Ration with and without Added Cystine.*

| Rat No. | Sex. | | Body weight. | Surface area. | Weight of hair. | | |
|---|---|---|---|---|---|---|---|
| | | | | | Total. | Per gm. body weight. | Per sq. cm. body surface. |
| | | | *gm.* | *sq. cm.* | *gm.* | *mg.* | *mg.* |
| 1 | ♂ | Control. | 93 | 190 | 0.7701 | 8.28 | 4.05 |
| 2 | ♂ | Cystine. | 98 | 196 | 0.8774 | 8.95 | 4.48 |
| 3 | ♂ | Control. | 99 | 198 | 0.7892 | 7.97 | 3.99 |
| 4 | ♂ | Cystine. | 109 | 209 | 1.1918 | 10.93 | 5.70 |
| 5 | ♂ | Control. | 83 | 178 | 0.7985 | 9.62 | 4.49 |
| 6 | ♂ | Cystine. | 95 | 193 | 1.1138 | 11.72 | 5.77 |
| 7 | ♂ | Control. | 88 | 184 | 0.6681 | 7.59 | 3.63 |
| 8 | ♂ | Cystine. | 105 | 205 | 0.9301 | 8 86 | 4.54 |
| 9 | ♂ | Control. | 109 | 209 | 0.7948 | 7 29 | 3 80 |
| 10 | ♂ | Cystine. | 127 | 229 | 1.0408 | 8.20 | 4.54 |
| 11 | ♂ | Control. | 96 | 194 | 0.8671 | 9 03 | 4.47 |
| 12 | ♂ | Cystine. | 113 | 214 | 1.2462 | 11.03 | 5.82 |
| 13 | ♀ | Control. | 89 | 185 | 0.9719 | 10.92 | 5.25 |
| 14 | ♀ | Cystine. | 100 | 199 | 0.9564 | 9.56 | 4.81 |
| 15 | ♀ | Control. | 92 | 189 | 0 9970 | 10 84 | 5.28 |
| 16 | ♀ | Cystine. | 109 | 209 | 1.2423 | 11.40 | 5.94 |
| 17 | ♂ | Control. | 187 | 289 | 3.0121 | 16.11 | 10.42 |
| 18 | ♂ | Cystine. | 210 | 310 | 2.9844 | 14.21 | 9.63 |

The purpose of the present study is to report the effects of the cystine supplement on the growth of the hair. The rats were killed with ether, skinned, and the skins depilated by enzyme digestion according to the method used by Lightbody and Lewis. The hair was collected, washed, dried, and weighed, also in accordance with their directions. The results are given in Tables I and II.

TABLE II.

*Growth of Hair on Potato Ration with and without Added Cystine.*

| Rat No. | Sex. | | Body weight. | Surface area. | Weight of hair. | | |
|---|---|---|---|---|---|---|---|
| | | | | | Total. | Per gm. body weight. | Per sq. cm. body surface. |
| | | | *gm.* | *sq. cm.* | *gm.* | *mg.* | *mg.* |
| 1 | ♂ | Control. | 102 | 200 | 1.1862 | 11.63 | 5.93 |
| 2 | ♂ | Cystine. | 115 | 216 | 1.7587 | 15.29 | 8.14 |
| 3 | ♂ | Control. | 94 | 192 | 1.1095 | 11.00 | 5.78 |
| 4 | ♂ | Cystine. | 121 | 224 | 1.6370 | 13.53 | 7.31 |
| 5 | ♂ | Control. | 96 | 194 | 1.2096 | 12.60 | 6.24 |
| 6 | ♂ | Cystine. | 120 | 224 | 1.7334 | 14.44 | 7.74 |
| 7 | ♂ | Control. | 109 | 209 | 1.3986 | 12.83 | 6.69 |
| 8 | ♂ | Cystine. | 123 | 225 | 1.2394 | 10.08 | 5.51 |
| 9 | ♂ | Control. | 124 | 226 | 1.3333 | 10.75 | 5.90 |
| 10 | ♂ | Cystine. | 145 | 247 | 1.4329 | 9.88 | 5.80 |
| 11 | ♂ | Control. | 102 | 200 | 1.2310 | 12.07 | 6.16 |
| 12 | ♂ | Cystine. | 115 | 215 | 1.4462 | 12.58 | 6.73 |
| 15 | ♂ | Control. | 80 | 174 | 1.0248 | 12.81 | 5.89 |
| 16 | ♂ | Cystine. | 109 | 209 | 1.4379 | 13.19 | 6.88 |
| 17 | ♀ | Control. | 125 | 227 | 1.5075 | 12.06 | 6.64 |
| 18 | ♀ | Cystine. | 129 | 230 | 1.9828 | 15.37 | 8.62 |

In analyzing the significance of these results, the differences in total weight of hair, and in weights per gm. of body weight and per sq. cm. of body surface (2), between pair mates have first been obtained. The mean of each set of eight or nine differences has then been computed, as well as the standard deviation. "Student's" method (3) has then been applied. In this method the ratio of the mean of the set of differences between paired observations to their standard deviation is obtained and is designated $z$. Then with the number of differences (eight or nine in this experiment) and the value obtained for $z$, one may enter "Student's" table and find the probability of getting a mean difference as large or larger than that obtained by the operation of chance only.

These calculations have been made for the data of this experiment, and the results summarized in Table III.

The positive signs of the average differences indicate that the rat fed cystine has produced the greater average weight of hair. The significance of these averages is measured by the values of $P$ in the last column of Table III. The smaller the value $P$ the less likely is it that the average difference in hair weight was the result of chance factors only, and hence the more significant it becomes as a specific effect of the cystine addition.

TABLE III.

*Estimation of Significance of Differences in Hair Weight between Pair Mates by Method of "Student."*

| Hair weight. | No. of differences. $N$ | Mean difference. $M$ | Standard deviation $s$ | Ratio $M:s$. $z$ | Probability. $P$ |
|---|---|---|---|---|---|
| Rats on garden pea ration. | | | | | |
| Total | 9 | +0.2127 | 0.149 | 1.43 | 0.0019 |
| Per gm. body weight | 9 | +0.80 | 1.49 | 0.54 | 0.084 |
| " sq. cm. surface | 9 | +0.65 | 0.77 | 0 84 | 0.023 |
| Rats on potato ration. | | | | | |
| Total | 8 | +0.3335 | 0.242 | 1.38 | 0.0041 |
| Per gm. body weight | 8 | +0 98 | 1.99 | 0.49 | 0.12 |
| " sq. cm. surface | 8 | +0.94 | 1.06 | 0.89 | 0.026 |

As regards total hair weight there can be no question but that the added cystine has favored a more rapid growth of hair, as it did of the total body tissues measured in body weight. The odds are only 2 and 4 in 1000 that chance produced the large average differences in total hair weight in the two experiments.

The average differences in hair weight per gm. of body weight between pair mates are evidently not significant. Chance alone could have produced the same result once in eight trials in the case of the experiment with potato, and once in twelve trials in the case of the the experiment with garden pea.

On the other hand, when the hair weight is computed per sq. cm. of body surface and the pairs compared on this basis, it appears that the average differences are of such size that chance could hardly have produced them. With $P = 0.023$, as in the first experiment, an average difference as large or larger than that obtained, +0.65, would have resulted from chance only once in forty-three trials. With $P = 0.026$, as in the second experiment, an average difference as large or larger than +0.94 mg. of hair per sq. cm. of surface would have resulted from chance only once in thirty-eight trials. These probabilities are so small that it becomes practically certain, especially on the combined weight of evidence of the two experiments, that the cystine added to the basal diet has produced a heavier coat of hair per unit of body surface.

## CONCLUSIONS.

The addition of cystine to a diet whose protein content is so low as to be the limiting factor in growth and is deficient in this amino acid, increases its value in the promotion of hair growth in the albino rat.

The coat of hair produced on such rations when supplemented with cystine is not only heavier *in toto*, as compared with that produced on the same amount of the unsupplemented ration, but it is heavier per unit of surface area. Hence it appears that, under these conditions, the growth of hair has been stimulated to a greater extent by the cystine supplement than has the growth of all tissues as measured by increase in body surface. There is no indication that the demands of the more vital tissues for cystine have taken precedence over the demands of the hair follicles.

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# EXPERIMENTAL HYPERPARATHYROIDISM IN GUINEA PIGS LEADING TO OSTITIS FIBROSA.*

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The relation of the parathyroids to the physiology and pathology of bone has been suggested in numerous studies. Clinical experience revealed the association of parathyroid enlargement with bone dystrophies. The significance of this association was brought out particularly in ostitis fibrosa cystica. Clinical symptoms, high serum calcium values and low serum phosphorus values, which are found in this condition, as well as clinical improvement following partial parathyroidectomy, pointed to hyperparathyroidism as the cause of the bone lesions observed.[1] Similar bone lesions, however, have not been reported to follow experimental hyperparathyroidism. Parathormone administration to dogs over a prolonged period resulted, indeed, in the production of negative calcium and phosphorus balances (1), but the depletion was quantitatively not great enough to be demonstrated by x-ray (2). Bauer, Aub, and Albright (3) administered parathormone over long periods of time to cats, rabbits, and rats, but only in the rabbit were they able to find definite effects—the thinning of the trabeculæ—without substantial effect upon the bone cortex. Histological examination of the bone was apparently omitted.

It has generally been concluded from the absence of effects or from relatively slight effects upon serum calcium after parathormone injections that cats, rabbits, rats, mice, and guinea pigs were relatively "immune" to parathormone. We believe that hypercalcemia should not be used, as it has frequently been used, both ex-

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* A preliminary report appeared in *Proc. Soc. Exp. Biol. and Med.*, **27,** 708 (1930).

[1] The literature on this subject has been reviewed by Barr and Bulger (Barr, D. P., and Bulger, H. A., *Am. J. Med. Sc.*, **179,** 449 (1930)).

perimentally and clinically, as the sole criterion of hyperparathyroidism. That a sufficiently increased rate of calcium excretion may be responsible for the absence of definite hypercalcemia has been suggested by Greenwald and Gross (1). Furthermore, parathyroid hypercalcemia may be absent or may be decreased after administration of bases (4), and therefore in herbivorous animals the dietary factor may be of importance. It may be taken as a starting point therefore that the effectiveness of parathormone is to be defined in terms of changes in acid-base equilibrium, with mobilization of the calcium reserves of bone and other tissues and increased calcium and phosphorus excretion, particularly in the urine. A very useful criterion of parathormone effect is available in the histological examination of the bones and tissues. While it can be generally used only to observe the end results of experimental hyperparathyroidism, and the quantitative estimate of bone resorption must be supplemented, if precise data are required, by chemical analysis, histological examination yields information not only on the extent of decalcification and resorption of bone, but also on the nature of the associated lesions in the bone and other tissues.

*The animals that have been assumed to be immune or tolerant to parathormone are of particular interest not only because of the mechanism by which hypercalcemia is prevented or moderated after single doses of parathormone, but also because of the magnitude of the effect upon the bone to be expected after large repeated doses of parathormone, which may be given without producing fatal hypercalcemia.*

In this study we used the guinea pig in an attempt to ascertain: (*a*) the effect of feeding and prolonged fasting upon its serum calcium and serum phosphorus responses to single injections of parathormone, and (*b*), the effects of repeated injections of parathormone (chronic hyperparathyroidism) upon the serum calcium and phosphorus as well as upon the bone and other tissues. The histological findings will be reported in detail elsewhere (5).

## *A. Effects of Single Large Doses of Parathormone on Fed and Fasted Guinea Pigs.*

Moritz (6) studied the effects of 50 units of parathormone, injected in several doses during a period of about 12 hours. The earliest significant rise was observed at about 10 hours after the first injection. High values (about 17 mg. per 100 cc.) were obtained as early as 10 hours and as late as 24 hours after the first injection. Return to normal values was obtained at intervals varying from 22 to 30 hours. The results show considerable variability between individual rabbits. No phosphorus data are given.

Collip (7), recalling his own earlier negative results in rabbits and rats, as well as those of Macleod and Taylor (8), stated that hypercalcemia could be induced in most normal rabbits by "enormous" doses of the extract, either in single or repeated injections. He found no effect on the inorganic phosphorus of the whole blood, and observed no signs of overdosage. Greenwald and Gross (1) stated that dangerous hypercalcemia could not be produced in rats and rabbits. They gave no specific data.

Condorelli (9) injected his own preparation of *canine* parathyroid gland into a dog and rabbits and obtained an elevation of serum calcium of only 0.8 mg. in the dog, as compared with elevations of 1 to 4.5 mg. in the rabbit. It is well known that to produce a given effect in the rabbit disproportionately larger doses of parathormone are required than in the dog. There was no such disparity between the dosages employed by Condorelli. Unless Condorelli's parathyroid extracts differed greatly from Collip's, his results must be viewed with reservations.

Tweedy and Chandler (10) found that rats responded to parathyroid extract by an elevation of serum calcium, and that parathyroidectomized rats were 2 to 3 times more responsive than normal controls. Süssman (11) was unable to get distinct elevation of serum calcium in mice, confirming a previous observation of Macleod and Taylor (8). Very high normal serum calcium values are reported by Süssman, 18 to 20 mg. per 100 cc. Macleod and Taylor do not give specific data.

Macleod and Taylor (8) stated that injection of massive doses of parathormone into guinea pigs, whether fasted or fed, produced "absolutely no effect." Later, Taylor (12) added the observation that "though a dosage greatly in excess of that given to dogs was employed, rabbits, guinea pigs, and mice were quite immune to the action of the extract."

We found no other studies on the blood calcium of guinea pigs, on the excretion of calcium, or on the exhaustion of their bone calcium after parathormone administration, either in single or repeated doses.

## *Experimental Procedures.*

*Diet.*—Most of the animals used were raised in the laboratory and were of clean and healthy stock. Their diet consisted of oats, hay, carrots, cabbage, and water *ad libitum.* When they were fasted they were put in clean cages with wide meshed bottoms to prevent consumption of feces. Fresh water was supplied during the period of fasting.

*Fasting.*—The normal diet of Herbivora, and specifically the diet of guinea pigs, yields in the course of metabolism a basic balance. However, during prolonged fasting guinea pigs excrete an acid urine. It seemed plausible that in fed guinea pigs the alkaline balance of the diet was at least in part responsible for their peculiar tolerance to large doses of parathormone. To test that assumption, we decided to eliminate the effect of the alkaline diet by prolonged fasting. It was judged necessary to institute long fasting periods (60 hours and longer), as well as short fasting periods of about 24 hours. The longer periods would prevent the possible effects of absorption during the passage of food through the gastrointestinal tract, and the delayed effects of recently absorbed inorganic salts. That excessive waste of tissues during prolonged fasting had not occurred was checked by observing the weight at the end of the fasting period. We found that the animals had lost not more than 10 to 15 per cent of their weight, after allowance was made for the weight of the gastrointestinal contents. On the whole, the younger animals had lost proportionately more weight than the adults.

Control tests on animals fasted for 60 hours or longer showed that equal periods of fasting, as such, did not affect their serum calcium. The effects of prolonged fasting on normal serum phosphorus were taken into consideration in interpreting the parathormone effects.

*Parathormone Dosage.*—The dosage of parathormone was varied between about 10 and 20 units per 100 gm. of guinea pig, on the basis of weight after fasting. When animals were fed up to the time of injection, their weight after fasting was estimated at 90 per cent of actual weight. This estimate was checked by weighing a large number of animals 24 hours after removal of food, and was found to be correct. The extract was injected subcutaneously, usually in the axilla, and frequently, when large doses were given, they were divided and injected into both axillæ.

*Collection of Serum Samples.*—Blood was taken at various intervals after the injection of parathormone in order to find the earliest evidence of definite effect on serum calcium and phosphorus. The animals were stunned by a sharp blow at the base of the skull and the large vessels of the neck were exposed and cut. The blood was allowed to clot and retract spontaneously before being centrifuged.

*Analytical Methods.*—Serum calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method and serum phosphorus by the Benedict-Theis method. Calcium determinations were performed in duplicate when possible. Phosphorus determinations were always duplicated.

## *Experimental Results.*

*Controls.*—For a proper interpretation of our results we found it necessary to define the range of serum calcium and phosphorus in normal guinea pigs, under our conditions. Thirty-three animals were examined.

Our average serum calcium value for all was 10.5 mg. per 100 cc. Each group average (young and old, fasted and fed) varied from that value only within the limits of analytical error. Only two animals exceeded that value by more than 1 mg. Three animals showed serum calcium as low as 9.5 to 9.8 mg. *We therefore feel justified in inferring parathormone hypercalcemia whenever a high proportion of animals shows more than 1 mg. rise above our average, for an extremely small proportion of normal animals shows a similar elevation above our average value* (Chart 1).

On the whole, the serum phosphorus tended to be higher in the younger than in the older animals, but there was considerable variability within each group. Furthermore, our experimental results in regard to phosphorus were variable and showed no uniform trend in response to parathormone injection. We have therefore not attempted to establish any strict normal criteria for serum phosphorus (Charts 2 and 3). There was practically no difference between the phosphorus values of the fed adults and those fasted 60 hours or longer (4 to 5 mg. per 100 cc. of serum). The young guinea pigs showed a lower serum phosphorus (about 5 to 6 mg.) after a prolonged fast when compared with the young fed animals or those fasted for about 24 hours (6 to 8 mg.).

*Effects of Single Doses of Parathormone on Serum Calcium. 1.*

*Young Guinea Pigs.*—In twenty-three young guinea pigs fasted for about 60 hours definitely higher serum calcium was found as early as 4 hours after injection; it increased to a maximum after 18 to 36 hours, and persisted at a high level to the 48th hour after the injection. On the other hand, twelve *fed* animals showed lesser effects at all intervals after a single injection (Chart 1). Although high calcium values were observed at the 24 and 36 hour intervals, substantially lower values were also found in each of these interval

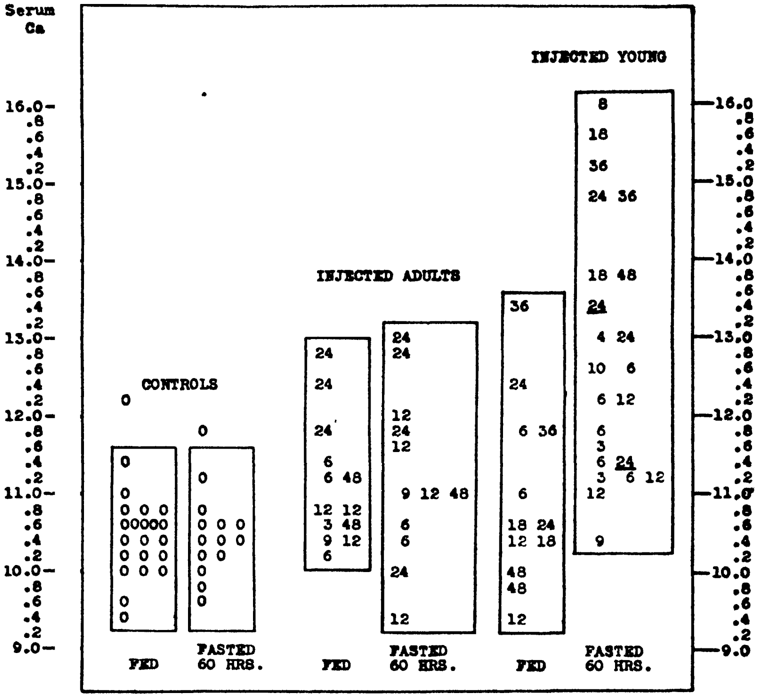

CHART 1. Range and distribution of serum calcium values at different intervals after a single injection of parathormone, compared with control values. The intervals (in hours) are represented by the figures. The distribution of control values is indicated by zeros; two control values not included within the boxes indicate, approximately, the rarity of occurrence of atypical values. Animals fasted for about 24 hours are not included in this chart. Interval figures of two animals without food for 41 hours are underscored.

groups. These latter values, very close to the normal range, corroborate our impression of the antagonism between the alkaline diet and parathormone effects upon the serum calcium of guinea pigs. (The serum calcium of ten young animals fasted for about 24 hours, at the various intervals after injection, resembled the fed group very closely. Their figures were therefore not included on the chart.)

Thus definite although not dangerous hypercalcemia follows administration of parathormone to young guinea pigs fasted for long periods, but is counteracted by feeding, completely after the short and long intervals, and partially even at the time of maximum effect—24 and 36 hours after injection. That prolonged fasting as such cannot be assumed to be a factor in raising appreciably the serum calcium of guinea pigs, apart from the action of parathormone, is shown by the values for the fasted controls, which are similar to those of the fed controls (Chart 1).

*2. Adult Guinea Pigs.*—Different results might have been expected with adult guinea pigs. The serum of fourteen fasted and thirteen fed animals was studied. The data are presented in Chart 1. The effects of parathormone upon the adult guinea pigs fasted for 60 hours or longer, the dose of extract being proportionate to weight, were not as great as upon the young animals. Nor is the effect of feeding on the serum calcium elevation in the adults as definite. These observations of the relatively slight effects of parathormone upon serum calcium of adult guinea pigs and of the relative unimportance of food in modifying such effects may explain the results of Macleod and Taylor (8), with both fasted and fed guinea pigs, if they used adults. Parathormone effects in adults would be observed only during a relatively short period and with insufficient dosage conclusively positive results could easily be missed.

As a general observation, it may be stated that a large dose of potent endocrine extract will frequently produce its greatest effect not by raising or depressing further the content of a chemical substance in the blood, but by the prolongation of such effect. Thus, maximum or minimum values of long duration may be the most striking evidence of the effect of a large dose in such tests. In our tests, a plateau persists between the 24 and 48 hour intervals in the young animals after a prolonged fast. Decline probably begins at about 48 hours. The absence of the plateau in the older, and its shorter duration in the fed young animals, is an added expression of less marked effects of parathormone upon the serum calcium.

*Histological Findings in the Bones.*—The bones of a number of *young* guinea pigs given single large doses of parathormone were examined, and the histological findings were consistent with the serum calcium data. Bones taken 18 hours after injection or later showed extensive resorption both of the cortical and spongy bone, proving histologically the removal of bone calcium, the mobilization of which was indicated by the high serum calcium. Fibrous transformation of the marrow occurred at the costochondral junction and in the vicinity of the epiphyseal cartilage plate. Guinea pigs killed 48 hours after a single dose of parathormone showed resorption of the bones so complete as to produce infraction. Often the cortex of the bone near the costochondral junction was converted into a pink-staining tissue, consisting of disorganized bone fibrils, which remained after the removal of the inorganic constituents of the matrix. These findings were striking evidence of the severe and extensive decalcification that one large dose of parathormone was capable of inducing. The decalcification was hardly apparent before the 12th hour and was, to some degree, more severe in the guinea pigs on a prolonged fast. *These changes were absent in adult guinea pigs.*

*Effects of Single Injections of Parathormone upon Serum Phosphorus.*—Although the range of normal phosphorus values in the young and adult, fasted and fed guinea pigs was wider than the range of calcium values, many phosphorus figures indicated overdosage. The effect on the *young* guinea pigs, fasted for about 60 hours was striking (Chart 3). A large proportion of values was far above the zone of similarly fasted controls. It is noteworthy that some of the high values were obtained at relatively earlier intervals after injection than the high calcium values. The *adults fasted for the same length of time* show few higher values than the fed adults after similar doses of parathormone. This is consistent with our observations on the effects upon serum calcium. On the other hand, the serum phosphorus of the *fed animals*, young and adult (Chart 2), shows a distribution not far above the zone of fed control values, indicating that parathormone action was moderated by the alkaline diet at least to the extent of mitigating or abolishing its *overdosage* effects.

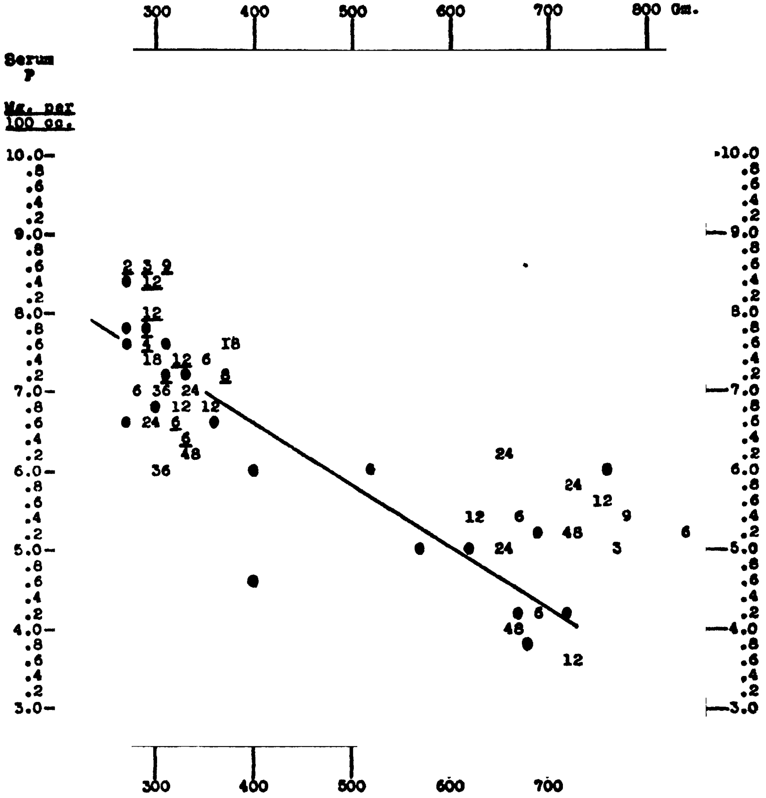

CHART 2. Serum phosphorus of fed animals, plotted against body weight. The distribution of phosphorus values obtained at different intervals (in hours, represented by the figures) after a single injection of parathormone is compared with the distribution of the control values (represented by black ovals). Underscored figures represent the intervals (after injection) for animals that were fasted for 24 hours. The sloping line indicates the approximate trend of control values. Values within about 1 mg. may be considered within the normal range.

High phosphorus values were frequently associated with high calcium values, although the reverse was not true: high serum calcium was sometimes found with apparently normal, or perhaps even low serum phosphorus. These results may be reconciled on the assumption that a coincidence of high calcium and high phosphorus indicates pronounced overdosage. As is well known, moderate doses of parathormone, in man and dog at least, lower the serum phosphorus, while overdosage will be followed in these species by high values. Collip (7) concluded that large doses of

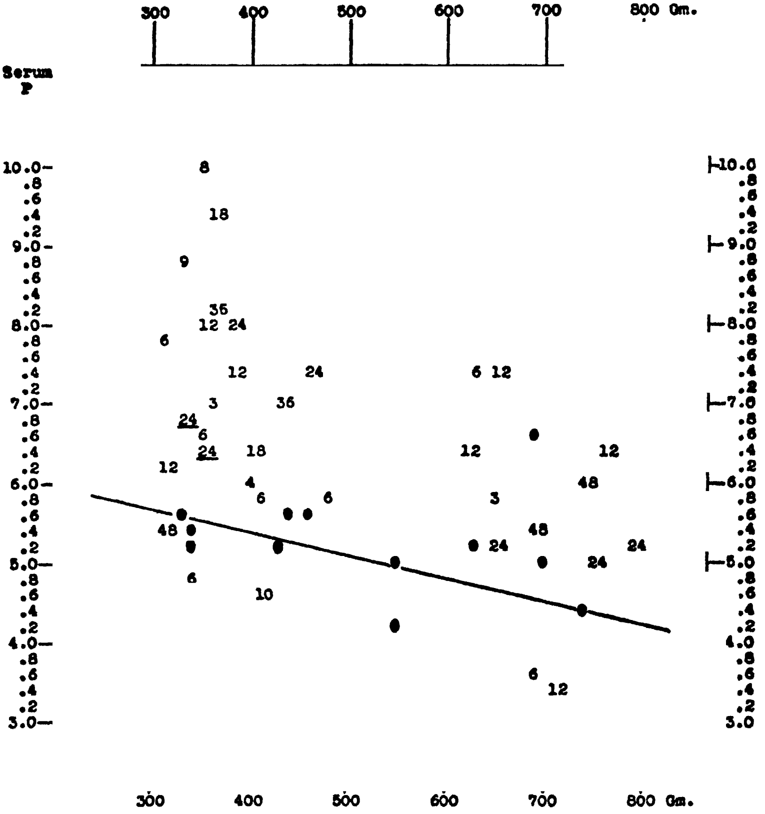

CHART 3. Serum phosphorus of animals after prolonged fasting, plotted against body weight. (For meaning of figures and black ovals see legend to Chart 2.) Underscored figures represent the intervals (after injection) for guinea pigs that were fasted for 41 hours. The other animals were fasted about 60 hours.

Note the depression of the serum phosphorus of controls.

parathormone produced no effects on serum phosphorus of rabbits. We believe that overdosage effects upon the serum phosphorus of *young* guinea pigs, and probably of rabbits may be demonstrated when the action of the extract is not inhibited or counteracted by the influence of an alkaline diet.

### *B. Effects of Repeated Doses of Parathormone.*

Few observations are recorded on the effect of injections repeated at intervals short enough to assure continuous action and continued long enough to insure considerable bone resorption.

Burns (13) injected gradually increasing doses of parathormone into eight rats—from 2 units on alternate days when the rats were about 6 weeks old to 10 units during the last 2 weeks of the test, which lasted for 14 weeks. Neither bone growth nor bone calcium were affected.

Hueper (14) and Learner (15) observed the effects of repeated injections of large doses of parathormone upon calcium deposition in tissues and organs. The bones were not examined histologically.

Bauer, Aub, and Albright (3) studied the effects of repeated parathormone injections into rabbits, rats, and kittens. They employed large doses, and continued the treatment for long periods. In rabbits they observed, at the termination of the experiment, an increased serum calcium and fewer spongy trabeculæ. They associated a negative calcium balance in both young and adult animals, and demands upon readily available calcium in both young and adult animals, with a depletion primarily of the spongy trabeculæ. They concluded that in cats and kittens parathormone had no effects, and that in rats its injection led to an increased number of trabeculæ. It is difficult to reconcile their observation of increased trabeculæ in these animals with their interpretation of the rôle of trabeculæ. They do not seem to have made a histological examination of the bones.

Lambie, Kermack, and Harvey (16) found that trabeculæ were thinned, bone ash decreased, but bone calcium not affected in rats after injection of 10 units daily for 21 days.

Normal or low serum calcium values, as we have pointed out, may not by themselves be taken as proof of absence of parathormone effect. This observation is particularly applicable when parathormone is administered repeatedly over a long period. In our experience with dogs (17), hypercalcemia was absent after prolonged administration of parathormone, unless the dose was increased or the animal had been allowed to replenish its reserves of available calcium. Mineral balance studies would be indispensable to establish the degree of effectiveness of parathormone during the course of administration, or histological examination of the bones, to establish the end results at the termination of the experiment. So far we have employed only the latter (18).

### *Experimental Procedures.*

The general procedures were detailed above.

The animals used in this test were all young, *this being considered a necessary condition for the production of maximum effects on the serum calcium as well as on the bone.* The initial weight of the guinea pigs was, with few exceptions, between 300 and 400 gm. The condition of the guinea pigs during the progress of the tests was checked by weighing at frequent intervals.

To test the effects of dosage and of length of treatment, the guinea pigs were divided into several groups: Group 1 included those given daily injections of 10 and 20 units for 10 and 16 days. Group 2 included three animals treated for 34 days; the maximum daily dose was 16 units, preceded in two of the guinea pigs by previous treatment with smaller doses. Group 3 included six animals given *rapidly increased* doses, up to 40 units daily for 6 days and four animals stepped up *gradually* to 60 units. Group 4 included animals given large doses—up to 100 units daily—for periods of varying duration, after preliminary treatment with smaller doses.

In several cases the guinea pigs were bled from the heart during the progress of the test, preferably before a dose was increased. The serum calcium and phosphorus, at any time during a course of daily injections, are under the influence of the last two or perhaps three doses injected. Therefore the interval between the last injection and bleeding is of little significance and will not be reported.

Our guinea pigs were bled less than 24 hours after removal of food, for we found that there were no marked differences after single parathormone injections between the serum calcium and phosphorus of guinea pigs fed up to the time of bleeding and of those fasted for 24 hours or less.

## *Experimental Results.*

Twenty-three guinea pigs were injected. All relevant data concerning them are summarized in Tables I to IV.

When receiving 10 units daily, or less, the animals were gaining weight. On about 20 units the weight generally rose in short tests. In our longest period of 34 days, with a daily dose of 16 units, the animals continued to gain until the 23rd day, then lost relatively rapidly until the 27th day, after which their weight remained practically stationary. On larger doses, loss of appetite was indicated by stationary or slightly decreased weight, and when the larger doses were continued, larger weight losses occurred.

*Serum Calcium.*—The effects of repeated injections of parathormone upon serum calcium varied with a number of factors, the most important being the quantity of parathormone administered within 48 to 72 hours before the chemical examination of the blood. The duration of treatment with a given dose modified its effect.

*Group 1.*—With doses of 10 units daily for 10 and 16 days, no clear evidence of parathormone effect on serum calcium was obtained. The average of five tests was 10.7 mg., which is within the normal range of calcium values; one value was 12.6 mg. On 20 units daily for 10 days, similar results were obtained in two animals (Table I).

*Group 2.*—On 16 units daily for 34 days a guinea pig tested at intervals showed little evidence of hypercalcemia. Definite hypercalcemia was obtained in two guinea pigs after 9 and 26 days on 16 units daily, preceded by treatment with smaller doses. In one of these animals no hypercalcemia was found after 16 days on 16 units daily, but after an additional 10 days the serum calcium rose to 13.9 mg. (Table II).

*Group 3.*—Six guinea pigs, given daily doses of 10 units for 2 days, 20 units for 2 days, and 40 units for 2 days, showed, with but one exception, definite effects on serum calcium. Four guinea pigs given slowly increasing daily doses up to 60 units showed a smaller effect (see Table III).

TABLE I.

*Effects of Moderate Daily Doses of Parathormone Administered for Short Periods.*

All analyses were performed at the termination of the tests.

| No. of animals. | Daily dose. | Days. | Ca | P |
|---|---|---|---|---|
| | *units* | | *mg. per 100 cc.* | *mg. per 100 cc.* |
| 2 | 10 | 10 | 12.6 | 6.9 |
| | | | 10.0 | 4.6 |
| 3 | 10 | 16 | 9.0 | 7.6 |
| | | | 10.7 | 6.7 |
| | | | 11.1 | 8.4 |
| 2 | 20 | 10 | 11.9 | 6.4 |
| | | | 11.1 | 4.7 |

One guinea pig on 20 units maintained its weight. The rest gained. Slight bone resorption and slight marrow fibrosis were found in all the animals examined.

TABLE II.

*Effects of Prolonged Administration of Moderate Doses of Parathormone.*

Analyses were performed at intervals on the days stated.

| No. of animals. | Daily dose. | Days | Serum analyses. | | |
|---|---|---|---|---|---|
| | | | Ca | P | Test day. |
| | *units* | | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| 1 | 16 | 34 | 12.1 | | 8th |
| | | | 11 2 | | 24th |
| | | | 9.6 | | 29th |
| | | | 11.6 | 6.4 | Last. |
| 1 | 2 | 8 | | | |
| | 8 | 17 | 10.1 | | 24th |
| | 16 | 9 | 16.7 | 6.0 | Last. |
| 1 | 4 | 8 | | | |
| | 16 | 26 | 11.0 | | 24th |
| | | | 13.9 | | Last. |

Regardless of presence or absence of hypercalcemia, bone resorption and slight fibrosis of marrow were found.

*Group 4.*—Three animals were injected 20, 40, and 60 units on 3 successive days. They died during the 3rd day before the serum calcium could be determined, but the bones showed marked resorption and decalcification. However, when similar or larger doses were employed after a gradual increase from smaller daily doses the animals survived for the duration of the experiment (up to 34 days), including 4 days on doses as large as 100 units. The elevation of serum calcium, while not proportional to the quantity of extract injected, was generally the greater the higher the final doses (see Table IV).

TABLE III.

*Effects of Rate of Increase of Daily Parathormone Dose.*

All analyses were performed at the termination of the tests. Serum Ca and P are measured in mg. per 100 cc.

| No. of animals. | Daily dose. | Days. | Serum Ca. | | | Serum P. | | |
|---|---|---|---|---|---|---|---|---|
| | | | Average. | Maximum. | Minimum. | Average. | Maximum. | Minimum. |
| | *units* | | | | | | | |
| 6* | 10 | 2 | | | | | | |
| | 20 | 2 | | | | | | |
| | 40 | 2 | 12.9 | 14.0 | 10.4 | 9.8 | 11.5 | 8.0 |
| 4† | 5 | 2 | | | | | | |
| | 10 | 4 | | | | | | |
| | 20 | 4 | | | | | | |
| | 30 | 4 | | | | | | |
| | 40 | 1 | | | | | | |
| | 60 | 1 | 11.9 | 12.5 | 11.0 | 6.7 | 7.6 | 5.5 |

* All the guinea pigs lost appetite; one was moribund on 6th day.

† Three guinea pigs gained weight. Marked bone resorption and fibrosis were found in all.

*Serum Phosphorus.*—Toxic hyperphosphatemia was found when large doses were given without previous periods of injection with smaller doses. The average serum phosphorus of six of the guinea pigs of Group 3 (on rapidly increasing doses) was markedly above the normal range (see Table III). On the other hand, animals treated for long periods with small doses, or with large doses after a previous period on smaller doses, tended to values lower than normal.

*Modification of Effects of Large Doses by Previous Treatment* *with Small Doses.*—The prevention of toxic hyperphosphatemia is not to be interpreted as an indication of the development of "immunity." We have actually observed the highest serum calcium values in our Group 4, in which large doses followed previous treatment with smaller doses (Table IV). However, due to a compensation mechanism, which remains to be elucidated, neither hyperphosphatemia nor an obviously moribund state was induced in these animals. In Group 3, on the other hand, although the calcium values were not as high, the toxic effect of large doses without previous treatment was indicated by the pronounced hyperphosphatemia and the obviously moribund state of one animal (Table III).

TABLE IV.

*Effects of Large Doses of Parathormone after a Long Period on Smaller Doses.* Analyses were performed at intervals on the days stated.

| No. of animals. | Daily dose. | Days. | Serum analyses. | | |
|---|---|---|---|---|---|
| | | | Ca | P | Test day. |
| | *units* | | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| 1 | 10 | 7 | | | |
| | 60 | 3 | 16.9 | 7.7 | Last. |
| 1 | 8 | 16 | 10.2 | | 16th |
| | 16 | 2 | | | |
| | 80 | 1 | | | |
| | 100 | 2 | 13.2 | | 21st |
| | 0 | 1 | | | |
| | 60 | 4 | 20.0 | 6 6 | Last. |
| 1 | 8 | 24 | 10 5 | | 8th |
| | | | 9.7 | | 23rd |
| | 30 | 1 | | | |
| | 40 | 1 | | | |
| | 80 | 1 | | | |
| | 100 | 2 | 12.5 | | 30th |
| | 0 | 1 | | | |
| | 100 | 4 | 18.5 | 6.9 | Last. |

Severe bone decalcification with fibrosis of the marrow was found in all.

*Histological Changes.*—A detailed account of the effects of repeated parathormone injections upon the structure of the bone will be published elsewhere (5). In general, repeated injection of even small doses of parathormone led *in young animals* to bone resorption with the appearance of Howship's lacunæ and osteoclasts on the resorption surfaces. Fibrous tissue appeared in the marrow, under the periosteum and endosteum and in the enlarged Haversian canals. This fibrous tissue contained numerous eosinophiles. The severity of the lesions was related to the dosage and to the duration of the experiment. After prolonged treatment, the lesions presented the typical picture of ostitis fibrosa.

## SUMMARY.

1. Control guinea pigs, under the conditions of the experiment, whether fasted or fed, young or adult, showed a serum calcium of about 10.5 mg. per 100 cc.; a very small proportion of the animals varied as much as 1.0 mg. above or below that figure. The serum phosphorus of fed controls showed the usual difference between young and adult animals. Values up to 8 mg. per 100 cc. are typical in the former, and values down to 4 mg. in the latter; in controls fasted for 60 hours or longer, the serum phosphorus of the young approached the values for the adult.

2. Young guinea pigs, *fasted 60 hours or longer*, showed the most distinct effects of single parathormone injections upon serum calcium. Serum calcium was definitely raised in some animals even at early intervals after injection, rose in all to a maximum at about 18 to 24 hours, and persisted at a high level until 48 hours after the injection. The serum phosphorus rose high above that of similarly fasted controls, indicating overdosage.

3. In young *fed* guinea pigs the serum calcium was definitely raised 24 hours after a single dose of parathormone, the effect persisting 12 hours longer, and disappearing afterwards. The phosphorus values fell mostly within the normal range, none being high enough to indicate overdosage, and some falling below the normal range—an effect similar to that obtained in the dog or man with moderate doses.

4. In adult guinea pigs, when fasted for 60 hours or longer, equal doses of parathormone on the basis of weight produced less marked effect on the serum calcium than in the young, at all intervals after a single injection. The serum phosphorus values were higher than normal, but not as far above the normal

range as in the young animals. They appeared earlier than the maximum effects on calcium.

5. In fed adult guinea pigs, distinct effects on the serum calcium were observed only at the 24 hour interval after a single injection. The serum phosphorus was slightly above the normal range.

6. Histological examination of the bones of *young* guinea pigs after single injections of large doses of parathormone showed severe bone lesions accompanied by decalcification. These changes were absent in the bones of *adult* guinea pigs.

7. After daily subcutaneous injections of 10 and 20 units of parathormone for about 2 weeks, young fed guinea pigs showed no clear effects on serum calcium or phosphorus. Histological examination of their bones showed evidences of slight resorption and decalcification.

8. On doses increased rapidly to 40 units daily, young guinea pigs showed hypercalcemia and hyperphosphatemia. One animal was obviously moribund. After a week or more on smaller doses, parathormone injections could be raised gradually to as high as 100 units daily without hyperphosphatemia, although with the higher doses serum calcium rose as high as 20 mg. Histological examination showed very severe resorption and decalcification of the bones, with marrow injury and the lesion complex characteristic of ostitis fibrosa.

## CONCLUSIONS.

1. In *young* guinea pigs the maximum effects of a single dose of parathormone upon serum calcium and phosphorus may be brought out after fasting for 60 hours or longer, presumably due to the removal of the normally basic diet.

2. The influence of the basic diet in preventing the maximum effects of a single dose of parathormone seems to be present in the adult guinea pigs as well as in the young, although to a lesser degree. (Other factors, such as an increased rate of excretion of calcium, may contribute to the tolerance of guinea pigs—and other herbivorous rodents—to parathormone, and are probably of even greater importance in explaining the resistance of rats, mice, and cats.)

3. Age seems to be a factor also in the production of bone lesions after single injections of parathormone. These are found in the young guinea pigs, but not in the adult.

4. Extremely large doses of parathormone may be administered daily to guinea pigs after previous administration of smaller and gradually increasing doses, without the development of extreme overdosage effects. The animals are thus enabled to survive for longer periods. With the increased duration of the experiment, lesions typical of ostitis fibrosa may be produced with certainty, with or without hypercalcemia, which depends largely upon the size of the dose. Even with smaller doses continued for a sufficient length of time bone resorption and decalcification may be produced although frequently hypercalcemia is absent.

The chemical determinations were performed with the assistance of Mr. A. Kibrick, Miss L. Hallman, and Miss R. Bonoff. The generous cooperation of Eli Lilly and Company, who supplied most of the parathormone used in these tests, is acknowledged.

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# STUDIES ON THE PLASMA CALCIUM-RAISING PRINCIPLE OF BOVINE PARATHYROID GLANDS.

## I. A METHOD OF PREPARATION AND SOME OBSERVATIONS ON THE YIELD, SOLUBILITY, AND STABILITY OF THE PRODUCT.

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### INTRODUCTION.

It has been definitely established during the past few years, that under appropriate conditions, a potent plasma calcium-increasing principle capable of affording a successful substitution therapy, can be extracted from bovine parathyroid glands. Collip (1) was first to offer convincing evidence, that its probable manner of action is through a direct effect on the calcium metabolism.

The work herein reported has to do with the yield, solubility, reactions, and stability relations of the active principle in the state of purity obtained by the author's modified method of preparation.

The more recent literature on parathyroid extracts is briefly summarized in so far as it appears to shed some light on the physical, or chemical properties of the hormone, or the substances intimately associated with it. Earlier literature has been adequately dealt with by Collip (1), and by Hjort, Robison, and Tendick (2).

### *Parathyroid Extracts.*

Collip (1) described an active serum calcium-increasing principle obtained by hydrochloric acid extraction of fresh parathyroid glands. The hormone is described (Collip and Clark) as a light gray, amorphous powder of the nature of a complex protein derivative, or else a substance intimately associated with such a type of compound. It was completely removed from acid solution by half saturation with ammonium sulfate, or complete saturation with sodium chloride. The substance contained sulfur and iron, but no phosphorus, or carbohydrate groups. Boiling 1 hour with either 10 per cent hydrochloric acid, or 5 per cent sodium hydroxide destroyed its physiological activity. It was also rendered physiologically inert by the action of both pepsin and trypsin.

Fisher and Larson (3) prepared extracts by heating fresh glands with normal hydrochloric acid. The stability of their product is indicated by the successful preservation of its physiological activity in neutralized solutions preserved with tricresol and kept in the ice chest for 3 months.

Hjort, Robison, and Tendick (2) obtained the hormone by aqueous and alcoholic hydrochloric acid extraction of bovine parathyroid glands. They state that the lipoid-free portion of the gland is the potentially active fraction.

Davies, Dickens, and Dodds (4) prepared extracts by acetone-picric acid extraction without preliminary hydrochloric acid extraction. The picrate of the hormone is described as insoluble in water, but convertible into the hydrochloride, which was found to be easily soluble.

Tweedy (5)[1] described a plasma calcium-increasing principle prepared by hydrochloric acid extraction of acetone-desiccated and defatted parathyroid glands. The active principle is described as adsorbable by kaolin at pH values greater, but not less than 5. Subsequent acidification to pH values of 1 to 3 failed to release the activity. In crude form the active material was found to be partially soluble, and stable in liquid ammonia and in ethyl lactate. 2 to 3 hours irradiation with ultra-violet light did not destroy, nor increase, the calcium-raising activity of the material.

In connection with the writer's extension of the above studies on purification of the parathyroid hormone some very striking instances of stability and unstability have been encountered. In particular, its great resistance to heat in phenolic solution, and

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[1] The publication referred to is an abstract of a dissertation presented by the author to the Department of Physiological Chemistry of the Ogden Graduate School of Science of the University of Chicago in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

its inactivation when subjected to the action of HCl gas in absolute alcohol appear to be significant. However, the interpretation of these findings must await a more intimate knowledge of the chemical nature of the active principle.

## EXPERIMENTAL.

### *Method of Preparation of Glandular Extracts.*

Fresh, frozen parathyroid glands, from which extraneous fat is trimmed, are ground in a meat chopper. The finely ground material is then transferred to a conical flask of suitable size, covered with 200 cc. of 3 per cent hydrochloric acid per 100 gm., and heated in a water bath at 70° for 20 minutes. The flask is immediately cooled under a stream of cold water, while being rotated in order that liquefied fat may cool and solidify on its inner surface. The acid extract is further freed of fat particles, and of undissolved glandular material by pouring through several folds of cheese-cloth. By means of weak sodium hydroxide the reaction of the solution is adjusted until near the neutral point, but slightly on the alkaline side of Congo red. An equal volume of redistilled anhydrous acetone is added, and the mixture set aside in the ice chest overnight.

The following day the aqueous acetone extract is filtered through a coarse filter paper, and evaporated *in vacuo* on a water bath at 50° until approximately one-third its volume is reached. It is then adjusted to the same volume as the neutralized acid extract by the addition of water and a sufficient volume of a freshly prepared solution of trichloroacetic acid to produce a concentration of 2½ per cent. After setting a few hours, or overnight in the ice chest the finely divided precipitate has settled to the bottom of the beaker.[2]

The trichloroacetic acid precipitate is separated from most of the supernatant solution by decantation, the remainder being removed by centrifugation. The tightly packed precipitate is stirred up with a little alcohol, transferred to a desiccator and

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[2] The supernatant trichloroacetic acid solution usually assumes a pink color, the intensity of which is increased on standing a few hours at room temperature. Some of the chromogenic material is carried down with the trichloroacetic acid precipitate, and is extractable therefrom by chloroform, but not by petroleum ether.

dried *in vacuo* at room temperature. After further drying for several hours over anhydrous calcium chloride, the material is ground to a fine powder. It is then tightly wrapped in an ashless filter paper, transferred to a Soxhlet extraction apparatus and extracted with redistilled anhydrous chloroform as long as lipid material is being removed.

When freed from chloroform, the material is found to be a gray, amorphous powder, partially or entirely soluble in 0.9 per cent sodium chloride in the concentration ordinarily used in potency testing (60 mg. per 5 cc.).

The solubility of the material in water depends upon the amount of hydrochloric acid carried down in the course of its preparation.

TABLE I.

*Yield of Active Material in Relation to Gross Weight and Counted Individual Glands.*

| Lot No. | Parathyroid glands. | | Yield of active fraction. | | |
|---|---|---|---|---|---|
| | Weight. | No. of glands. | | | Yield per gland. |
| | *gm.* | | *gm.* | *per cent* | *mg.* |
| 65 | 364 | 764 | 0.9170 | 0.25 | 1.2 |
| 66 | 276 | 612 | 1.2200 | 0.44 | 2.0 |
| 67 | 284 | 614 | 1.6000 | 0.56 | 2.9 |
| 68 | 878 | 2266 | 3.4700 | 0.39 | 1.5 |
| 69 | 512 | 1270 | 3.0019 | 0.58 | 2.3 |

If not entirely water-soluble, it can be rendered so by suspending it in 4 or 5 times its volume of acid alcohol (prepared in the ratio of 15 cc. of 10 per cent HCl to 85 cc. of 95 per cent alcohol) in which it is partially soluble. Ether is then added to complete precipitation of the soluble fraction, and the material is washed free of alcohol with ether at the centrifuge. This treatment converts the product into a white powder, now entirely soluble in 0.9 per cent sodium chloride in the amount ordinarily used.

## *Yield of Active Material.*

Parathyroid glands, collected and trimmed of extraneous fat at the abbatoir vary considerably in fat content, and can hardly be expected to be entirely free of foreign tissue. Inasmuch as they also vary in size, and probably in their individual content of the active principle, or fraction bearing the active principle, it is surprizing that the yield of the active fraction is as uniform as is shown in Table I.

As indicated below the average yield is approximately 0.44 per cent when calculated on the basis of gross glandular weight. When such material as has been used, is desiccated and lipid material as completely removed as possible, it is reduced to about one-tenth the gross moist weight. Calculated on a dry basis the active fraction would constitute about 4 to 5 per cent. In the writer's opinion one would expect the active principle itself to constitute a much smaller per cent of the protein portion of the gland.

### *Method of Biological Assay.*

Normal dogs of either sex have been used as animals on which to test the potency of various preparations. In the writer's experience the response of the same dog to the same effective dose of a given preparation is quite constant. Hence, the comparative strength of two preparations has been estimated by testing them on the same animal.

The animal was fed during the morning of the day the test was begun. Uneaten food was then removed from the cage, and food was not supplied again until after the second blood sample had been taken. Under these conditions, it has been observed in control experiments that the second calcium value is very seldom higher than the first.

The material tested was dissolved in physiological salt solution, injected subcutaneously, and blood drawn from a leg vein 15 to 16 hours later. Coagulation of blood samples was prevented by means of heparin.

Calcium estimations were made by the Kramer-Tisdall method (6) as modified by Tweedy and Koch (7). By this modification the accuracy has been increased by washing the precipitated calcium oxalate three times with 0.5 per cent ammonia water saturated with calcium oxalate. Titrations were conveniently made by use of a special micro burette (8).

A calcium increment as measured from normal values to 14 to 16 mg. appears to be a more accurate indication of the potency of a dose than where values of 18 to 20 mg. are obtained (Table II).

As Collip (1) has pointed out the latter values represent the maximum physiological response to one dose administered subcutaneously, and therefore a quantitative relationship between mg. of dose administered and increase in blood calcium does not hold when the physiological limit is closely approached.

### *Solubility of Active Material.*

The active principle has been found to be completely soluble in warm 90 or 100 per cent phenol, and also in orthocresol. Similarly to Abel and Geiling's (9) experience in the precipitation of insulin from phenol solution, it has been found possible to remove parathyroid hormone from phenol solution (1) by the addition of ether, and (2) by the addition of several volumes of water. Preparations such as described here have been further purified by removing a less active fraction from phenolic solution by means of alcohol, followed by precipitation of the more active fraction by ether. In certain instances the active fraction has been freed of phenol by steam distillation.

TABLE II.

*Potency of Active Material.*

| Dog No. | Weight. | Parathyroid hormone. | | Blood plasma Ca. | |
|---|---|---|---|---|---|
| | | Lot No. | Weight of dose. | Initial value. | After 15 to 16 hrs. |
| | *kg.* | | *mg.* | *mg. per 100 cc.* | *mg. per 100 cc.* |
| 1 | 11 3 | 65 | 60 | 11.00 | 14.40 |
| 1 | 11 3 | 66 | 60 | 12.00 | 15.20 |
| 2 | 12.0 | 67 | 60 | 10 00 | 13.00 |
| 3 | 17 1 | 67 | 60 | 12 70 | 15 10 |
| 4 | 16 0 | 68 | 60 | 10 60* | 18 20 |
| | | 68 | 30 | 11.20 | 13.30 |
| 5 | 15 9 | 68 | 120 | 11.40* | 19.25 |
| 6 | 14.0 | 68 | 60 | 10.75 | 16 85 |
| 7 | 13 6 | 68 | 60 | 12.10 | 17 15 |

* 22 hours afterward, the calcium values were 15.6 and 18.5 mg. respectively.

The active material is insoluble in anhydrous acetic acid, but is appreciably soluble in 98 per cent, and freely soluble in 94 per cent acetic acid. It is almost completely removed from this solution by the addition of 2 to 3 volumes of acetone.

Although completely insoluble in 100 per cent pyridine, it has been observed that if the material is first dissolved in 10 per cent acetic acid, it is not precipitated therefrom by pyridine even when the concentration in pyridine is gradually increased up to 94 per cent.

The solubility of various preparations in formamide varies considerably. Active material has been precipitated from solution in formamide by acetone, but precipitation by butyl alcohol yields an inactive fraction. Although the active principle is partially soluble in glycerol warmed to 50° it has not been recovered therefrom.

Furthermore the active material has been found to be insoluble in ethyl formate, ethyl butyrate, carbon tetrachloride, benzene, and methyl salicylate, absolute methyl alcohol, and absolute ethyl alcohol.

### *Stability in Phenolic Solution.*

60 mg. of active material (P68), which had been heated for 1½ hours at 120° in phenol in an atmosphere of nitrogen produced a 60 per cent increase in the blood calcium of a 21 kilo dog. Several experiments have demonstrated that as the temperature is increased above 120° there is a progressive loss in activity concomitant with a destruction of sulfur-containing material.

### *Further Observations on Stability.*

Untreated glands kept in the frozen state for 6 weeks yielded the usual amount of the active fraction.

Acetone-dehydrated and defatted glands stored in a desiccator over anhydrous calcium chloride for 1 year retained practically their original content of the active principle. Parathyroid hormone prepared by the method described, and similarly stored for 8 months has been found to be equally as potent as when first prepared. Freshly prepared material, when exposed to a temperature of 100° in an electric oven for 10 days was found to have retained about half its activity.

Active material contained in a quartz tube, tightly closed by a rubber stopper, was completely inactivated after 36 hours continuous irradiation by a Cooper Hewitt mercury vapor arc.[3]

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[3] The Cooper Hewitt mercury vapor arc used in these experiments was furnished through the courtesy of the Victor X-Ray Corporation.

## SUMMARY.

1. Potent preparations of parathyroid hormone in dry form in quantities of 1 to 3 mg. per gland may be separated from hot hydrochloric acid extracts by removal of inert material with acetone, followed by trichloroacetic acid precipitation of the active fraction, and removal of inert lipoid material from this fraction by chloroform extraction.

2. The product as prepared is usually soluble in water and 0.9 per cent sodium chloride to the extent of 0.5 to 1 per cent at room temperature, but if it is not that soluble it can be rendered so by suspension in aqueous acid-alcohol and precipitation therefrom with ether.

3. The active principle is insoluble in anhydrous acetic acid, but appreciably soluble in 98 per cent, and freely soluble in 94 per cent acetic acid. Although insoluble in pyridine, it is not precipitated from solution in 10 per cent acetic acid by pyridine when the concentration of the latter is gradually increased to 94 per cent.

4. It is approximately 10 per cent soluble in dry phenol, or orthocresol at 70°. After heating for 7 hours in dry phenol at 70° the activity is unaltered. When heated at 150° for 1 hour only partial inactivation results. There is complete loss of activity on heating the phenolic solution at 175° for ½ hour in an atmosphere of nitrogen.

5. Complete inactivation results when the active material is suspended in 0.5 per cent HCl in absolute alcohol and heated for 20 minutes at 70°, or when it is suspended for 1 hour in absolute alcohol, saturated with hydrogen chloride gas at 10°.

A portion of the materials used in the above studies was purchased from a grant by the Committee on Scientific Research of the American Medical Association. The author also wishes to thank Armour and Company for their cooperation in supplying properly prepared glands, and Doctor F. C. Koch for his criticism of this paper.

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# A SYNTHESIS OF TRYPTOPHOL.

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Felix Ehrlich demonstrated that yeast attacks the natural amino acids essentially by splitting off carbon dioxide and replacing the amino group with hydroxyl. By this reaction, the greater part of fusel oil is derived from the leucines. In the case of tryptophane, the process gives rise to tryptophol or 3-indole ethyl alcohol, first described by Ehrlich in 1912. Since then, the substance has engaged the attention of various investigators of intermediary metabolism (*cf.* Guggenheim and Löffler, 1915–16; Ward, 1923; Jackson, 1929). Notwithstanding this background of biological interest, there appears to be no recorded synthesis of tryptophol. It seemed worth while, therefore, to attempt such a synthesis with the object both of producing the substance itself and of extending the somewhat limited procedures available for the construction of compounds in the indole series. The writer has succeeded in securing tryptophol in a good yield and in a high state of purity through the reduction of indole acetic acid ester by means of sodium in alcohol. The properties of the synthetic product show that it is identical with the tryptophol prepared by the yeast decomposition of tryptophane according to the directions of Ehrlich.

## EXPERIMENTAL.

*Indole Acetic Acid.*—This compound was prepared in good yield according to Majima and Hoshino (1925). Indole magnesium iodide was treated with chloroacetonitrile to produce 3-indole acetonitrile which on hydrolysis gave the 3-indole acetic acid. After purification it melted at 164–165° (corrected).

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* Seessel Fellow, Yale University, 1930.

*Methyl and Ethyl Esters.*—Both esters were readily secured in practically a quantitative yield in the usual way by refluxing the acid with a considerable excess of the proper absolute alcohol containing a little dry hydrochloric acid gas, followed by evaporation of the alcohol, washing of the ether solution of the ester with sodium bicarbonate solution and with water, drying of the ether solution with calcium chloride, and finally, vacuum distillation. Both esters distilled in the neighborhood of 180° at 2 mm. of pressure. Neither ester could be induced to crystallize.

*Reduction of the Ester. Experiment 1.*—8.5 gm. (0.045 mol) of the methyl ester were reduced in methyl alcohol (dried with sodium) with the proportions of solvent, sodium, and toluene and the use of mechanical stirrer as directed by Marvel and Tannenbaum (1922). The yield of crude product, which did not crystallize, amounted to 3.0 gm. (0.019 mol). This was converted to the picrate which was recrystallized from hot water and then subjected to alkaline decomposition and ether extraction. The evaporated ether extract was crystallized from dilute alcohol and then from ether-petroleum ether. The pure white crystals melted at 58° (corrected) and exhibited no melting point depression when mixed with a specimen of tryptophol prepared from tryptophane according to Ehrlich's method.

*Experiment 2.*—It was subsequently learned[1] that very good yields of certain dihydric alcohols could be obtained by simply adding very dry alcoholic solutions of the esters to the sodium. The experiment described below was performed in similar fashion. Commercial absolute ethyl alcohol was treated (*cf.* Smith, 1927) with sodium sufficient to react with all the water present and then in addition with enough ethyl phthalate to react with all the free alkali remaining in the solution. The alcohol was refluxed ½ hour and then distilled, to the amount of 250 cc., directly into the flask in which the reduction was to be carried out. To this alcohol, 9.15 gm. (0.045 mol) of dry ethyl indole acetate were next added, followed by 15 gm. (0.65 mol) of sodium. The flask was attached

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[1] Private communication from Dr. Wallace H. Carothers of the Experimental Station of E. I. du Pont de Nemours and Company. Dr. R. H. F. Manske of Yale University has confirmed Dr. Carothers in this finding, and further has found ethyl phthalate a good inexpensive ester to use in preparing the alcohol according to the method of Smith (1927).

to a reflux condenser equipped with a calcium chloride tube, and after ½ hour, heated for 2 hours on the steam bath. A little remaining sodium was dissipated by the addition of a small amount of 50 per cent alcohol and the product worked up in the customary fashion. Direct crystallization from benzene-petroleum ether yielded 4.33 gm., melting at 57° (corrected) and 1.30 gm. melting at 54–57° (corrected). An additional 0.25 gm. was obtained as the picrate. The total of 5.88 gm. amounts to a yield of 81 per cent of the theoretical. If the 0.70 gm. of indole acetic acid recovered is taken into consideration, the yield is 89 per cent of the theoretical. The product was purified by vacuum distillation and crystallization from benzene-petroleum ether to give beautiful white glistening plates. As an aid to comparison, the physical properties of various specimens of tryptophol and its derivatives are given in Table I.

TABLE I.

*Properties of Tryptophol and Derivatives.*

| Tryptophol sample. | M.p. | M.p. of picrate. | M.p. of phenyl urethane. | Nitrogen. |
|---|---|---|---|---|
| | *°C.* | *°C.* | *°C.* | *per cent* |
| (*a*) Ehrlich | 59* | 94–96† | | 9.02 (Dumas.) |
| (*b*) From tryptophane according to Ehrlich (*cf.* Jackson, 1929) | 58–59‡ | 100–101§ | 130–131§ | 8.62 (Kjeldahl.) |
| (*c*) Synthetic (Experiment 2) | 58–59‖ | 100–101§ | 130–131§ | 8.61 ( " ) |
| (*d*) Mixed melting points, (*b*) and (*c*) | 58–59 | 100–101 | 130–131 | 8.69 (Theory.) |

All melting points are corrected.

The solvents employed are indicated by symbols:

* Ether-petroleum ether.

† Water.

‡ Dilute alcohol.

§ Benzene.

‖ Benzene-petroleum ether.

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# THE DETERMINATION OF INORGANIC SULFATE IN SERUM.

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At the meeting of the Society of Biological Chemists, in 1927, a micro method for the determination of inorganic sulfate in serum was described by the author (1). This method involved the removal of protein with trichloroacetic acid, the precipitation of sulfate with benzidine, and the determination of benzidine in the precipitate by a colorimetric oxidation technique. It was based in part upon the nephelometric methods of Denis (2), and Denis and Reed (3), and in part upon the method of determining sulfate in urine described by Fiske (4). As far as the author knows the colorimetric method used was new. No extensive bibliography will be presented, as one is included in a recent article by Wakefield (5).

The method described in 1927 was studied by Wakefield (5), modified by him in some particulars, and adapted, not only to the determination of inorganic sulfate, but also of total sulfate in serum and of inorganic and total sulfate in urine. The work upon the method in this laboratory has led to the development of a technical procedure somewhat different from that recommended by Wakefield. Furthermore certain difficulties met with when the method has been applied by others have been rather extensively investigated. It seems worth while, therefore, to present our results at this time.

The discussion can conveniently be divided into two parts. The first of these concerns the plan followed in precipitating sulfate with benzidine and the second the technique used in developing the color. Fiske (4) described the difficulties which are encountered when benzidine is used to separate sulfate quantitatively from other inorganic compounds. The substances which caused difficulties were phosphates and chlorides. He found that if the acidity of the solution in which the precipitation is carried out is low, phosphates are carried down, and that sodium chloride, if present in high concentration, increased the solubility of benzidine sulfate. The amounts of blood used in the determination do not contain sufficient sodium chloride to affect the results, as was shown repeatedly, but the satisfactory separation of very small amounts of sulfate from very large quantities of phosphate was a problem which required much study. High concentrations of benzidine tended to cause a precipitation of phosphate and low ones not to precipitate completely very small amounts of sulfate. Variations in acid concentration had the opposite effect, as high concentrations tended to hold benzidine sulfate in solution and low ones to permit the precipitation of phosphate. The strengths of solutions finally adopted permitted the quantitative precipitation of very small amounts of sulfate (sulfate equivalent to 0.001 mg. of S was regularly recovered) and prevented the precipitation of phosphate in amounts equivalent to 1 mg. of P. When a similar technique was used in some studies of experimental nephritis, the values obtained for sulfate were obviously too high. Investigation showed that when both sulfate and phosphate are present in high concentrations some of the phosphate is carried down with the sulfate. For example, solutions containing 0.5 or more mg. of sulfur gave high values if 1.0 mg. of phosphorus was present, but if the amount of phosphate were equivalent only to 0.5 mg. of phosphorus, or if sulfate was present in lower concentration, the theoretical amount and no more was recovered. It is not difficult to avoid this error by using less material for the determination when both phosphate and sulfate are present in very high concentrations. It is also possible to dissolve the precipitate first obtained in acid and reprecipitate the benzidine sulfate, thus separating it from the contaminating benzidine phosphate. Our experiments seem to suggest that extreme conditions of this kind are rare in clinical nephritis, for in the most severe case of this kind studied by us identical values were obtained after one and after two precipitations with benzidine. When the analysis was carried out in one laboratory upon material obtained after hydrolysis of tissues with hydrochloric acid a white crystalline precipitate, quite unlike benzidine sulfate formed. This was probably benzidine chloride, which is not very soluble in acetone. Under such conditions it is obvious that the method cannot be used. Since 16 mg. of sodium chloride give no precipitate under the analytical conditions recommended for 1 cc. of serum this difficulty can hardly apply to the determination of sulfate in blood.

In studying the development of color from benzidine solutions it was found essential to use the same concentrations of acid and of oxidizing reagents in all cases if the depth of color was to be kept proportional to the amount of benzidine present. It was found also that different proportions were best adapted for different ranges of concentrations of benzidine. The difficulty met with when large amounts were present was the formation of brown pigment which precipitated easily. The technique finally adopted gives yellow colors which reach their maximum intensity in 10 minutes, and which remain proportional to the amounts of benzidine present for more than half an hour. The color obtained from benzidine equivalent to amounts of sulfur ranging from 0.0005 to 0.05 mg. are strictly proportional to each other as far as such a relationship can be determined by the colorimeter. If more than 0.05 mg. of sulfur is present it is necessary to work with a smaller amount of the sample.

Many precautions were taken in standardizing the method. Pure potassium sulfate was added to serum in amounts varying from 0.001 to 1.0 mg. equivalent of sulfur and was recovered quantitatively. Treatment with benzidine after barium chloride had been used to remove sulfate from serum gave no precipitate. Working as rapidly as possible after proteins had been thrown down with trichloroacetic acid gave the same results as were obtained when the filtrate was allowed to stand overnight before it was analyzed. Results upon solutions equivalent to 1 and to 0.5 cc. of serum were proportional. Repeated washing with acetone did not dissolve any of the precipitated benzidine sulfate. The only difficulties that seemed to be met with were the necessity for using aliquots if the sulfate content of the blood was high and the occlusion of some phosphate when both sulfate and phosphate are present in very large amounts. The method recommended for carrying out the determination is described below.

### *Reagents.*

Reagent 1 was a 20 per cent solution of sulfate-free trichloroacetic acid in sulfate-free water. This acid may be prepared by redistilling a good commercial product at reduced pressure (5).[1] Reagent 2 was pure benzidine hydrochloride for standards.[1] Make a stock solution by dissolving 4.015 gm. in a liter. 1 cc. of this is equivalent to 0.5 mg. of sulfur. Dilute to give solutions with 1 cc. equivalent to 0.1, 0.01, and 0.001 mg. of sulfur. These are fairly stable, but should be rejected if they develop much color. Reagent 3 was 1 per cent solution of good grade benzidine[1] in high grade acetone.[2] Reject when the solution becomes highly colored. It is best to prepare it daily. Reagent 4 was the same grade of acetone for washing. Redistil if the solution is colored or contains inorganic material. Reagent 5 was hydrochloric acid in approximately N and 0.2 N concentration. Reagent 6 was U.S.P. 3 per cent hydrogen peroxide freshly diluted with 9 parts of distilled water. Bottles which have been opened for a long time should not be used. Reagent 7 was 2.5 per cent ferric chloride[3] in distilled water. All glassware must be clean and free from sulfate.

*Method.*—Precipitate the protein by adding 1 part of 20 per cent trichloroacetic acid to 1 part of serum. Centrifugalize. Precipitation of the sulfate is done by adding 2 cc. of the supernatant liquid (equivalent to 1 cc. of serum) to 5 cc. of 1 per cent benzidine in acetone in a sharp-pointed, conical, 15 cc. centrifuge tube; mix; cap. Let stand at least 15 minutes and centrifugalize at high speed. Longer standing up to 2 hours does not affect the determination. It is possible to cause the precipitate to "flock out" by placing it in ice water for an hour or more. This makes the determination possible when a powerful centrifuge is not available. Decant, drain for 5 minutes, and wipe the inside of the lip of the tube. Wash with 15 cc. of acetone, taking care to break up the precipitate. Centrifugalize, decant, drain, and wipe the lip of the tube as before. The washing may be repeated, but this is not usually necessary.

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[1] The Eastman Kodak Company has furnished these reagents of a satisfactory degree of purity. If trichloroacetic acid is ordered from them it is necessary to specify a "sulfate-free" or a distilled product.

[2] Baker's analyzed reagent was satisfactory.

[3] Merck's Blue Label product was used.

*Solution of Benzidine Sulfate.*—Add 2 cc. of 0.2 N hydrochloric acid and warm. If the amount of sulfur present is less than 0.5 mg. the precipitate will usually dissolve easily, and the material is ready for the final step in the determination described below. If the precipitate does not dissolve add 4.6 cc. of N hydrochloric acid, warm if necessary, and make up to 25 cc. with distilled water. Measure 2 cc. into a 15 cc. centrifuge tube for the final determination.

*Colorimetric Determination.*—Prepare standards containing benzidine equivalent to from 0.001 to 0.05 mg. of sulfur. These standards should be so chosen that none is more than twice as strong as the next lower one. Add to each 2 cc. of 0.2 N hydrochloric acid and dilute to 10 cc. Dilute the unknown, containing 2 cc. of 0.2 N hydrochloric acid as described above, to 10 cc. Add to each of the standards and unknown solutions 1 cc. of the freshly diluted hydrogen peroxide. Mix. Then add to each 0.5 cc. of 2.5 per cent ferric chloride solution and mix again. Let stand 10 minutes and read in a colorimeter within the next half hour.

*Calculation.*—$\frac{S}{U} \times$ sulfur equivalent of standard = sulfur (as sulfate) in 1 cc. of serum; where $S$ = reading of the standard, and $U$ = reading of the unknown. If the dissolved precipitate was made to 25 cc. the result should be multiplied by 12.5. If more than 0.7 mg. of sulfur is present in 1 cc. of serum it is usually necessary, because of an interfering brown pigment, to repeat the determination upon a smaller aliquot of the 25 cc. of the benzidine sulfate solution. In this case add 0.2 N hydrochloric acid to make the total volume up to 2 cc. before diluting for the color comparison. If such a smaller aliquot is taken the appropriate factor must be used in making the calculation. The final figure may be multiplied by 3 if it is desired to express the results as sulfate, and by 100 to convert them into terms of mg. per 100 cc.

## CONCLUSION.

A colorimetric method for the determination of sulfate in serum is described and some of the precautions which must be taken to insure correct results in unusual conditions discussed.

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# LIPEMIA IN HEMORRHAGIC ANEMIA IN RABBITS.

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(Received for publication, June 3, 1930.)

Milne (1912–13) observed that repeated bleeding of rabbits, at intervals of a few days, lowered the hemoglobin percentage to less than 30, and produced lipemia in these animals. Under such conditions the serum sometimes contained as much as 10 per cent of ether-soluble fats. Milne, in agreement with Boggs and Morris (1909), attributed this lipemia, which was simultaneous with an accumulation of fats in other organs, to the lowered oxidation resulting from the decrease in hemoglobin. In 1917, Ellermann and Meulengracht noticed the same lipemia in anemic rabbits, and demonstrated that its occurrence in such rabbits could be prevented by fasting before and during the bleeding period or by reinjection of the serum withdrawn after removal of the corpuscles.

As yet there is no thoroughly satisfactory explanation of how this lipemia comes about, and no information as to its eventual connection with certain other changes occurring at the same time in rabbit blood—changes in the concentration of the serum proteins and in the colloid osmotic pressure. In order to elucidate these questions and, eventually, to demonstrate a definite relation between the appearance of lipemia and the changes in the percentage of serum proteins and the colloid osmotic pressure, I have repeated the experiments of Ellermann and Meulengracht. In other experiments, I have varied the technique to ascertain additional points that might, perhaps, prove of interest in this connection.

While my research was in progress, Fishberg and Fishberg (1927–28, 1928) reported some experiments upon the same problem. Their findings, which were for the most part in agreement with my results, showed that a fall in the hemoglobin percentage was accompanied by a fall in the protein concentration, together with a lowering of the colloid osmotic pressure in the serum, coinciding with an increase in the serum lipoids. Fishberg (1929) considered the lipemia to be a compensatory measure of the organism, brought about by the falling colloid osmotic pressure; for the author found a higher colloid osmotic pressure in lipemic serum taken during the experiment than in other rabbit serum where the same protein concentration was obtained by simple dilution.

### *Technique.*

As a rule, the blood used for these experiments was taken from a vein in the ear of the rabbit. The hemoglobin percentage was determined with the Autenrieth-Köningsberg apparatus; the lipoid content was obtained by Bing and Heckscher's (1924, 1925) method; and the protein concentration in the serum was found by means of Pulfrich's refractometer. In a few of the last determinations, difficult because of the serum turbidity, control tests were made by Kjeldahl's method, in order to check the accuracy of the results. In each case, however, the two results were in agreement. The colloid osmotic pressure was determined by Krogh and Nakazawa's (1927) method. During the experiments the rabbits were kept on a fixed diet consisting of cabbage, carrots, and a few oats.

A typical experimental protocol is given in Table I.

It will be noticed in Table I, that after 3 days the hemoglobin percentage fell to about 28, and the colloid osmotic pressure to about 280, and that from then on there were practically no changes in these values.

After a few days the serum gradually became opalescent, and there was an increase in the amount of blood fat, but only to about 3 times the lowest value observed. The rabbit was apparently perfectly well in spite of being bled about 400 cc. in 2 weeks; that is to say, in this period the animal lost more than twice its total amount of blood, reckoning the blood to be 5 per cent of the body weight.

In three rabbits an effort was made to increase the degree of lipemia by bleeding, washing the red cells in physiological saline, and reinjecting them. This effort was unsuccessful, the results in regard to the amount of lipemia being the same as in simple hemorrhage.

## SUMMARY.

Five rabbits were made anemic by daily bleedings. After a few venesections the hemoglobin percentage fell to about 30, and at about the same time there was a fall in the protein percentage and in the colloid osmotic pressure in the serum of the animal.

TABLE I.

*Lipemia Data for a 3.5 Kilo Rabbit.*

| Date. | Blood drawn. | Hemoglobin. | Serum. | | | Blood lipoids. | Appearance of serum. |
|---|---|---|---|---|---|---|---|
| | | | Total protein. | Colloid osmotic pressure. | Colloid osmotic pressure per gm. protein. | | |
| | *cc.* | *per cent* | *gm. per cent* | *mm. $H_2O$* | *mm. $H_2O$* | *mg. per 100 cc.* | |
| Dec. 17 | 40 | 65 | 7.2 | 322<br>335 | 46 | 11 | Clear. |
| " 18 | 30 | 47 | 6.6 | 286<br>305 | 46 | 8 | " |
| " 19 | 20 | 32 | 6.3 | 257<br>272 | 42 | 8 | " |
| " 20 | 35 | 28 | 6.8 | 270<br>280 | 41 | 9 | Slightly opalescent.<br>" " |
| " 21 | 20 | 28 | 7.0 | 280<br>288 | 41 | 14 | " " |
| " 22 | 25 | 28 | 7.2 | 275<br>281 | 39 | 19 | Opalescent. |
| " 23 | 35 | 28 | 7.2 | 286<br>279 | 40 | 23 | " |
| " 24 | 25 | 29 | 6.9 | 281<br>285 | 41 | 20 | " |
| " 25 | 15 | 27 | 7.2 | 278<br>290 | 40 | 11 | Slightly opalescent. |
| " 26 | 35 | ? | 6.8 | 286<br>290 | 43 | 10 | Opalescent. |
| " 27 | 35 | 28 | 6.6 | 265<br>253 | 40 | 11 | " |
| " 28 | 40 | 26 | 6.8 | 252<br>238 | 36 | 20 | " |
| " 29 | 20 | 24 | 7.2 | 270 | 38 | 19 | " |
| " 31 | 25 | 20 | 6.9 | 280 | 41 | 15 | Slightly opalescent. |

During the rest of the experimental period the values of the hemoglobin percentage, the protein percentage, and the colloid osmotic pressure did not show any marked change.

The onset of the lipemia was coincident with the lowest colloid osmotic pressure, but otherwise the amount of serum lipoids was found to vary somewhat, evidently depending upon the fat depots in the animal. During the experiments the largest amount of serum lipoids was a little over 1 per cent, which is a considerably smaller value than was found by Milne (1912–13) in his experiments. It may be that my rabbits were smaller and thinner than the rabbits employed by Milne, and that this may explain the difference in the intensity of the lipemia.

It was noticed that, after the colloid osmotic pressure fell to a value somewhat above 200 mm. of water, it tended to remain at this level in spite of the continuous removal of large amounts of proteins from the organism. At the same time there were but very slight changes in the protein percentage. These facts are peculiar and they do not agree very well with the view that forms a part of the modern theory of edema; namely, that in nephrosis the great loss of proteins by way of the urine lowers the protein percentage and the colloid osmotic pressure so much that edema may ensue. In the production of edema, there must be other factors more significant than the fall in protein percentage and in colloid osmotic pressure from loss of proteins, since in nephrosis the loss of proteins by way of the urine is comparatively much smaller than the loss of proteins produced in the rabbits of this experimental series by means of venesection, and these animals show but a slight decrease in the protein percentage and in the colloid osmotic pressure of the serum, and no edema.

Though I obtained about the same results as Fishberg (1929) from similar experiments, I would not agree with her conclusion that lipemia represents "an effort of the organism to compensate for the decrease in osmotic pressure." One can only say that lipemia is about coincident with low colloid osmotic pressure.

Practically the same results were obtained in three other experiments with rabbits to remove the serum, the washed corpuscles being reinjected intravenously. Otherwise, the technique was the same as in the preceding experiments.

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# A METHOD FOR THE ESTIMATION OF THE ACID-BASE BALANCE IN THE ASH OF PLANTS.*

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(Received for publication, June 23, 1930.)

The common method of determining the excess of acidic or basic elements present in the ash of plant or animal tissue is to make a rather complete analysis of the material, computing the excess from the results of this analysis. Such a procedure is difficult, long, and the results secured are subject to the errors which may appear in the individual determinations.

The advantage of a method which would give an index of the quantity of excess acidic or basic elements present in a plant material without such a laborious procedure as a total analysis is obvious. Extreme accuracy could be sacrificed, if necessary, for speed, inasmuch as small differences in total acidity or basicity in a food material are not as important in many cases as the relative final reaction of the ash elements.

Sherman and Sinclair (1), in a preliminary report, and Sherman and Gettler (2) in a later paper discuss the significance of differing amounts of acidic and basic elements in the ash of foodstuffs, and give results of analyses of a large number of materials used as foods. In this work the authors point out the fact that most of the current compilations of analyses give results in terms of the whole material, whether it be animal or vegetable, and not of the edible portion. The method discussed in the present paper has been applied only to plant materials, and in most cases to the entire plant. However, there seems no reason why this method could not be applied to the edible portions of plants, and perhaps to materials of animal origin.

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It is, then, with the idea that this method may have a wider application than is discussed in this paper, that the procedure described below is presented.

The simplest method for determining the excess of acidic or basic elements in the ash of food materials is to titrate a solution of the ash obtained from a given sample of the material directly with standard acid or alkali. It was early recognized, however, that such a procedure was inaccurate, since a great part of the more volatile elements are lost during ignition. For lack of a better method this procedure is still in use, however, for as late as 1929 Morse (3) titrated the ash of cranberries directly with standard acid, in order to express the alkalinity of the ash of the fruit.

In devising a method which will give a satisfactory measure of the excess of acidic or basic elements, the first consideration is that the more volatile elements be retained while the organic matter is decomposed by ignition. The elements which are lost in considerable quantity seem to be sulfur and chlorine. It is known that the greater part of the former element present in plant or animal tissue is in organic combination, while chlorine has been shown to be liberated from its compounds when ignited in the presence of carbon (4).

It has been shown by Benedict (5) and others that in order to hold organic sulfur during ignition, relatively large amounts of a basic element must be present, together with a suitable oxidizing medium. He has found such a combination of properties in copper nitrate, which, when used in combination with ammonium nitrate and sodium chloride, according to the procedure of Denis (6) allows the retention of organic sulfur in the ignition of many substances. Since it was shown by Frear (7) that such a medium could be used to retain the sulfur of certain plant materials, it was felt that the combination of reagents recommended by Denis (6) and stated above might retain the chlorine as well, and thus give an ash on which a true acid-base ratio could be determined.

Determinations of the content of sulfur and chlorine before and after ignition with Benedict-Denis solution showed practically quantitative retention of these two elements. The precipitate of copper hydroxide formed during the titration, however, readily adsorbs materials in solution. Since this adsorption prevents accurate titration, it was necessary to abandon this line of attack.

A search for a more suitable reagent which would not yield a colored solution or a highly adsorptive precipitate upon neutralization led to the selection of magnesium nitrate as the basis for a solution which would have sufficient oxidizing power, and still contain a suitable alkaline element. The nitrate radical present functions as a strong oxidizing agent, while the magnesium furnishes comparatively strong alkali. A tentative method for the determination of sulfur is given in the Methods of Analysis of the Association of Official Agricultural Chemists (8) which retains that element by the use of magnesium nitrate. Hartwell, Smith, and Damon (9) in work which was the forerunner of the present investigation used calcium nitrate solution in an attempt to determine the acid-base balance in plant material but the reaction upon ignition was extremely violent, and analysis of the resulting ash showed losses of sulfur and chlorine. Work was thus undertaken to establish the suitability of magnesium nitrate as a reagent to retain during ignition the elements sulfur and chlorine present in plant material.

## EXPERIMENTAL.

From the standpoint of ease of manipulation, the amount of reagent chosen must be sufficiently large to allow complete ignition at low temperature, but not so large as to cause an explosion or even an extremely violent reaction to take place. Various concentrations of magnesium nitrate solutions were used, and from this preliminary work it was evident that a solution of 25 gm. of magnesium nitrate ($Mg(NO_3)_2 \cdot 6\ H_2O$) made to 100 cc. with distilled water was suitable, when used in the proportion of 25 cc. of solution to 2 gm. of finely ground plant material. This was entirely from the physical point of view, and it remained to determine the efficiency of the reagent in retaining sulfur and chlorine.

During the following work the procedure given below was followed, and is the method recommended.

### *Method.*

Weigh 2 gm. of finely ground plant material into a porcelain beaker. Add to this about 10 cc. of distilled water, with care, so that no mechanical loss occurs. After the entire mass of the plant material has been wetted, add 25 cc. of a solution containing

25 gm. of magnesium nitrate per 100 cc. Mix, and place on an electric hot plate regulated so that the mix simmers but never boils violently. Allow the liquid to evaporate, heating in all about 3 to 4 hours.

When the residue is entirely dry, but not so dry as to become explosive (this degree of drying is best regulated by experience), remove from the hot plate, cover, and place in an electric furnace heated to about 250°. After 15 minutes, or when it is evident that the first reaction is over, raise the temperature of the furnace to 500°. This heating may be prolonged if black particles of unburned carbon are in evidence, but ordinarily requires not more than 30 minutes. The temperature should never be greater than 500°. Remove from the furnace when the contents of the beaker are white or nearly so, and allow to cool. Add about 10 cc. of water cautiously, allowing the ash to become thoroughly wet. Add 60 cc. of normal nitric acid from a burette, and digest on a hot plate just below the boiling temperature for 3 hours. Transfer the solution and any insoluble residue to an Erlenmeyer flask and titrate the excess acid with normal sodium hydroxide, using methyl red indicator.

Blank determinations are made in the same manner as above, 1 gm. of sugar being used in the place of the plant material.

### *Calculation.*

The amount of normal acid used minus the amount of normal alkali gives the amount of acid required to neutralize the alkalinity of the crop, plus that of the magnesium oxide formed from the oxidizing solution. The amount of normal acid required to neutralize the blank determination is then subtracted from the total quantity of acid used, and the result in cc. equals the milli-equivalent weight of excess alkalinity in 2 gm. of plant material. Should the material have an excess of acidic elements, some of the alkali normally neutralized by the blank will remain. Thus the result of the above calculation will be negative, indicating an excess of acidic elements in the ash.

Qualitative tests for escaping sulfur or chlorine were made by igniting plant material treated with the above reagent and dried, under a close-fitting hood made from a glass funnel. All fumes arising from the ignition were drawn by suction, first through a

¼ inch copper tube heated to redness, and finally through a series of wash bottles filled with distilled water. After the ignition was complete, the contents of the wash bottles were tested qualitatively for sulfates and chlorides. The absence of sulfates was noted in practically every case, while traces of chlorides were found in most of the solutions, although the quantities were very minute.

Quantitative determination of sulfur in four samples of ground plant material were made before and after ignition with the magnesium nitrate. The analysis of the original material was carried out by the adaptation of the Benedict-Denis method described by Frear (7). Results of these determinations are given in Table I. Similar determinations of chlorine before and after ashing were made by the method of the Association of Official Agricultural Chemists and are shown in the same table.

TABLE I.

*Comparison of Sulfur and Chlorine Present in Plant Material and in Ash of Same Material after Ignition with Magnesium Nitrate Solution.*

| Crop. | S in original material. | S in ash. | Cl in original material. | Cl in ash. |
|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* |
| Corn | 0.103 | 0.092 | 0.161 | 0.196 |
| Buckwheat | 0.094 | 0.083 | 0.901 | 0.939 |
| Cabbage | 0.928 | 0.960 | 1.391 | 1.378 |
| Spinach | 0.438 | 0.416 | 0.787 | 0.772 |

From the data shown, it is evident that the losses in these two elements are small. The discrepancies which appear are all within a reasonable limit of error.

There remained to determine whether the value obtained by the titration of the excess of basic or acidic elements was comparable with that calculated from a chemical analysis of the stronger acid-forming and base-forming elements of each of these crops. Analyses of the four samples of plant materials for the elements chlorine, sulfur, phosphorus, potassium, calcium, magnesium, and sodium were accordingly made. All of these were determined by the official methods of the Association of Official Agricultural Chemists, with the exception of sodium, which was determined by the zinc-sodium-uranyl acetate method of Barber and Koltoff (10).

The results of this comparison are given in Table II. The results secured by the titration method are in good agreement with those obtained by the complete analyses. Only the elements mentioned were considered in the calculation of the acidity or basicity of the ash. Other elements, such as iron, manganese, aluminum, and silicon, are not included in the calculations, since these elements are relatively weakly acidic or basic and with the exception of silicon, are present in very small amounts. Silicon has such extremely weak capacity for neutralizing strong bases that there seems little need for including it in the calculation. To a considerable extent these weak acidic and basic elements balance, thus compensatiing for any error.

TABLE II.

*Milli-Equivalents of Strong Acid-Forming and Base-Forming Elements in 100 Gm. of Dry Plants, and Their Algebraic Sum, Compared with the Results Obtained by the Titration Method.*

| Crop. | Cl | $SO_4$ | $PO_4$* | K | Na | Ca | Mg | Excess of basic elements by analyses. | Excess of basic elements by titration. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *m.-eq.* | *m.-eq.* |
| Corn | 4.5 | 6.6 | 20.3 | 30.5 | 2.0 | 11.6 | 16.3 | 29.0 | 25.0 |
| Buckwheat | 25.4 | 6.8 | 18.5 | 38.8 | 2.7 | 53.6 | 26.9 | 71.3 | 71.3 |
| Cabbage | 36.7 | 64.5 | 22.4 | 58.1 | 27.6 | 169.4 | 19.7 | 151.2 | 148 8 |
| Spinach | 22.2 | 28.4 | 66.3 | 154.2 | 82.5 | 14.6 | 46.3 | 180.7 | 190.9 |

* $H_3PO_4$ considered as a dibasic acid.

The determination of the acid-base balance of the ash by the ignition in the presence of magnesium nitrate can be made in approximately 5 hours, and a considerable number can be conducted simultaneously, thus allowing the results to be secured in a fraction of the time required for the analytical procedures.

## SUMMARY.

A method for the estimation of the excess of acidic or basic elements in the ash of plant materials is presented. The procedure requires much less time than a complete analysis for the elements concerned. It is shown that sulfur and chlorine are retained when plant material is ignited in the presence of magnesium nitrate. The acid-base balance of the plant, determined by titration of this ash, is in good agreement with the stoichiometric balance of the amounts of strong acidic and basic elements obtained by chemical analysis.

The author wishes to express his obligations to Mr. John B. Smith for his advice throughout the course of this work.

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# QUANTITATIVE DIFFERENTIATION OF VITAMINS A AND D. II.*

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(Received for publication, July 1, 1930.)

In a previous paper from this laboratory (1) the experimental data presented on this subject were discussed in their bearings both upon the precautions which should be taken in measurements of vitamin A values, and the interpretation of such work as had been done in this direction in our own laboratories before the recognition of vitamin D as a separate growth essential.

Steenbock and Nelson (2) had found the growth of their young rats, when placed on a diet adequate so far as known except for fat-soluble vitamins, to be first inhibited by the depletion of their bodily surplus of vitamin D; when this latter factor was supplied by irradiating the rat or its food, growth was resumed until the vitamin A stores were depleted, as evidenced by the incidence of ophthalmia as well as by cessation of growth. Relative to their needs, these young rats had a larger bodily reserve of vitamin A than of vitamin D. Growth ensued only when both factors were supplied.

In the work of this laboratory, when dry whole milk constitutes one-sixth and wheat five-sixths of the food mixture, the diet (Diet A) is found adequate for the normal nutrition of the rat throughout successive generations (3, 4), while if the proportion of milk is increased to one-third of the wheat and milk mixture, even better nutrition results (3); and the young from the diet containing the higher proportion of milk possess at every age larger bodily reserves of both vitamins A and D, as indicated by the fact that they require a longer time (and make more growth) upon vitamin A-free or vitamin D-deficient diets, respectively,

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before their bodily stores of these vitamins are sufficiently depleted (or "diluted" by growth) for gain in weight to cease.

Workers in this laboratory had for some years regarded the young rats from the better diet (Diet B) as more suitable for use in vitamin A determinations than those from Diet A; Sherman and Burtis (5) found them less susceptible to infection during the test period, even though just before the test period the difference in the bodily store of vitamin A was eliminated by means of a preliminary depletion period. The larger proportion of milk in the family dietary may also furnish the young with important reserves of other factors whose importance is not yet clearly recognized. As a rule, animals from Diet B have larger bodily stores of vitamin D than of vitamin A relative to their needs (1, 6, 7). We know, however, that there are variable amounts of vitamins A and D in milk powder, which is the source of these vitamins for both the mothers and the young animals up to the time that the latter are taken for test. Hence we employ in the work of this laboratory measures to provide vitamin D liberally in all vitamin A experiments (1, 6, 8) in accordance with the general principle of conducting quantitative determinations of vitamins under conditions which provide amply for all known nutritional essentials except the one under investigation. Additional vitamin D can readily be furnished to experimental rats by incorporating in the basal diet some form of irradiated ergosterol.

Recently Sherman and Burtis (6) have described some refinements of the method for determining vitamin A, as developed in this laboratory (8) from the basic work of Drummond, Coward, and their associates. The method now involves the use of animals of accurately known age and nutritional history. Rats reared by mothers receiving our Diet B, with or without 30 to 60 gm. of fresh lean beef per adult per week, are placed when 3 or 4 weeks old upon a basal diet excellently adapted to the needs of the animals in all other respects but carefully freed from vitamin A. The basal diet which we now use consists of casein (freed from fat-soluble vitamins) 18 per cent, salt mixture (9) 4 per cent, dry brewers' yeast 10 per cent, sodium chloride 1 per cent, cornstarch 67 per cent, irradiated commercial cholesterol 0.05 per cent, or an equivalent amount of some other form of irradiated ergosterol. The rats are kept on this diet until growth has ceased because of depletion of their bodily stores of vitamin A. Young rats from our colony, receiving this basal diet usually gain about 35 to 50 gm. in body weight before growth is suspended. When growth has definitely ceased under conditions which extended experience indicates to mean the depletion of the bodily store of vitamin A, the animal usually also showing other signs of vitamin A deficiency, each animal is placed in a separate all-metal cage with raised screen floor to prevent access to excreta, and the test period or experimental period proper is begun.

For this work we limit the choice of experimental animals to rats weighing from 70 to 100 gm. at the end of the preliminary period, because the rate of gain on a given allowance of vitamin A tends to vary with the size of the individual.

Usually, of each litter of animals used in work of this kind, one or more is continued on the basal diet without supplement as a (negative) control which is observed daily for symptoms of the characteristic pathology of vitamin A deficiency. The experience thus gained ultimately enables the worker to detect these symptoms in their incipiency and thus to begin the test period when the animals have been thoroughly depleted of surplus vitamin A but are not in a seriously pathological condition. From this point onward, the other test animals of a litter are fed different amounts of the food under investigation. To judge the rate of gain permitted by a given amount of food, an average of the gain in gm. made by representative animals from several litters should be used. The daily allowance of a food material which is required to support an average rate of gain of 3 gm. per week during the test period is considered to furnish 1 unit of vitamin A. When quantitative studies of the effect of some treatment on vitamin A are being made, it is recommended that carefully matched rats from the same litters be fed in parallel such daily amounts of the treated and untreated preparations as have been shown by preliminary experiments to contain 1 unit of vitamin A. The extent of inactivation can be calculated from the amounts of test material necessary to permit similar growth.

In an analysis of almost 200 experiments in which the rate of gain was approximately our standard unit rate of 3 gm. per week, it was found that to have shortened the test period from 8 weeks to 5 weeks would have resulted in only slightly less regular findings and a somewhat higher average rate of gain per week. The shorter test period therefore tends to give the material tested a higher numerical rating in vitamin A value. Studies are in progress to determine, if possible, the explanation for this. The shorter test period may prove advantageous in avoiding complications arising from other factors in these measurements of vitamin A, as we now believe may be the case in similar measurements of vitamins B and G.

The relationship between vitamin intake and gain in weight is not always linear. Therefore, ideally, comparisons of the vitamin content of foods should be made on the basis of the amounts necessary to produce some selected average rate of gain in a sufficient number of suitably standardized rats in well controlled experiments. In our experience 3 to 4 gm. per week is the most satisfactory rate of growth upon which to base comparisons of the relative amounts of vitamin A in test materials. This rate of growth is low enough to permit the results to be verified by means of the larger and smaller gains in weight resulting from feeding correspondingly larger or smaller amounts of the vitamin A-containing food. Also at this rate of growth males and females may be used interchangeably. Probably too, the lower the rate of growth the less likely it is to be influenced by factors other than vitamin A.

When such amounts of two or more vitamin A-containing products are fed that the attendant rates of growth are decidedly different from each other and from 3 to 4 gm. per week, their relative vitamin potency can be compared only by establishing, or assuming as established, the relationship between various rates of growth and graded allowances of vitamin A and then interpreting the findings on the basis of such a set of standards. Such a set has been tentatively proposed by Batchelder (10) who controlled all the factors known to affect the accuracy of vitamin A determinations.

Hundreds of experiments have shown that when all the conditions affecting the accuracy of the determination are observed, the rat growth method gives reproducible results for the determination of vitamin A. In our experience the growth response under the conditions here described is a more reliable quantitative index of vitamin A than other physiological responses.

When rats from our Diet B are fed a diet adequate otherwise so far as known but decidedly deficient in vitamin D, they continue to gain, on the average, from 6 to 8 gm. per week for about 3 months (in one series for as long as 4 months) before their growth is suspended (7), whereas when given a diet otherwise adequate but free from vitamin A, cessation of growth usually occurs in 4 to 5 weeks.

While this relatively larger store of vitamin D served to protect, for the most part, our determinations of vitamin A before the recognition of vitamin D as an essential nutritional entity, it has made our animals unsatisfactory as reagents for determining vitamin D by the rat growth method. On a basal diet consisting of casein (carefully freed from fat-soluble vitamins) 18 per cent, dry brewers' yeast 10 per cent, Osborne and Mendel salt mixture 4 per cent, corn-starch 66 per cent, sodium chloride 1 per cent, and dried spinach 1 per cent, vitamin D appears to be the first growth-limiting factor for our rats up to about 150 to 180 days of age. However, this diet does not support a fully normal rate of growth even when vitamin D is liberally supplied. The increased rate of gain observed when vitamin D is abundantly supplied as contrasted with that resulting when vitamin D is limited to the preexperimental bodily store (augmented with possible traces in the basal diet) is too small to encourage attempts to use growth as a quantitative measure of supplementary vitamin D, although we find that graded portions of a vitamin D-containing food (whole summer milk powder) produce corresponding increments in weight. However, as we pointed out in a previous paper (7), we are deterred from using gain in weight as a measure for vitamin D not only because of the comparatively small difference at any age in the rate of gain between rats receiving only the basal diet and those receiving this diet liberally supplemented with vitamin D, but also because the measurements would have to be made at a level of growth rather high for precise determinations.

From our data we believe that if rats are reared by mothers on a diet such as Steenbock and Nelson (2) employed—relatively high in vitamin A but low in vitamin D—they might quickly be depleted of their store of vitamin D and then respond quantitatively by growth to graded amounts of vitamin D-con-

TABLE I.

*Co P n of Several Criteria for Expressing Calci on of Bon in Four Series of Experiment*

| Series. | Preexperimental period. | Experimental period. | | Green femur. | | | | | Dried extracted femur. | | | | | Ash. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | No of rats | Ash. | | Ca | | No. of rats. | Ash | | Ca | | No. of rats | Organic residue, ratio. |
| | | | | | Mean. | P E.* | Mean. | P.E.* | | Mean. | P.E.* | Mean. | P E * | | |
| | days | days | | | per cent | | per cent | | | per cent | | per cent | | | |
| A | 0 | 28 | Positive control group. | 17 | 26 5±0.20 | | 9.69±0.09 | | 17 | 53.6±0.36 | | 19.6±0 14 | | 17 | 1.16±0.016 |
| | | | Negative " " | 36 | 20.6±0.19 | | 7.72±0 07 | | 36 | 47.8±0.32 | | 17.8±0.12 | | 36 | 0.92±0.011 |
| | | | Difference. | | 5.9±0.28 | | 1.97±0.11 | | | 5.8±0.48 | | 1.8±0.18 | | | 0.24±0.019 |
| | | | Critical ratio.† | | 21 | | 18 | | | 12 | | 10 | | | 13 |
| B | 0 | 35 | Positive control group. | 14 | 28.1±0.36 | | 10.45±0.14 | | 14 | 59.4±0.34 | | 22.1±0.11 | | 14 | 1.47±0.019 |
| | | | Negative " " | 20 | 22.5±0.27 | | 8.31±0.10 | | 30 | 54.0±0.33 | | 20.0±0.13 | | 20 | 1.17±0.020 |
| | | | Difference. | | 5.6±0.45 | | 2.14±0.17 | | | 5.4±0.47 | | 2.1±0.17 | | | 0.30±0.028 |
| | | | Critical ratio. | | 12 | | 13 | | | 12 | | 12 | | | 11 |
| C | 24 | 28 | Positive control group. | 11 | 30.9±0.57 | | 11.70±0.25 | | 11 | 59.3±0.52 | | 22.5±0.26 | | 11 | 1.46±0.031 |
| | | | Negative " " | 13 | 25.6±0.34 | | 9.52±0.13 | | 13 | 53.2±0.45 | | 19.8±0.23 | | 13 | 1.15±0.024 |
| | | | Difference. | | 5 3±0.66 | | 2.18±0.28 | | | 6.1±0.69 | | 2.7±0.35 | | | 0.31±0.039 |
| | | | Critical ratio. | | 8 | | 8 | | | 9 | | 8 | | | 8 |
| D | 31 | 28 | Positive control group. | 19 | 29.3±0.30 | | 10.96±0.16 | | 18 | 58 1±0.31 | | 21.7±0.14 | | 19 | 1.40±0.018 |
| | | | Negative " " | 13 | 20 4±0 52 | | 7.41±0.18 | | 13 | 47.7±0.79 | | 17.4±0 30 | | 13 | 0.93±0.030 |
| | | | Difference. | | 8 9±0 60 | | 3 55±0 24 | | | 10.4±0 84 | | 4 3±0.33 | | | 0.47±0.035 |
| | | | Critical ratio. | | 15 | | 15 | | | 12 | | 13 | | | 13 |

* P. E. indicates the probable error.

† The term critical ratio is here used to indicate the ratio of the difference to its probable error.

taining food. With our rats, which possess a higher reserve of vitamin D than do theirs, we find the extent of calcification a more satisfactory measure (than growth) of graded allowances of vitamin D as supplements to a basal diet containing vitamin A and a uniform and adequate supply of the inorganic elements. This appears to be true both when the experimental period is begun immediately upon separation of the test animals from their mothers or after attenuating the bodily surplus of vitamin D by feeding a diet otherwise adequate but deficient in vitamin D for a prolonged period. At each of the ages studied—56 days, 80 days, and 155 to 180 days—the increase in the percentage of calcium in fresh femurs was proportional to the intake of vitamin D. We are reporting our results on the basis of the percentage of calcium (or of ash) in the fresh femur, because it is less time-consuming and, as shown by the extensive data summarized in Table I, is quite as precise a measure as other criteria commonly employed.

The percentage of calcium (or of ash) in the bones of animals receiving the basal diet and any definite amount of vitamin D varies considerably from individual to individual and from litter to litter. However, in five series of experiments, the averages of groups receiving graded amounts of vitamin D, each group within a series including animals from the same litters and strictly comparable so far as weight, age, and sex were concerned, show graded differences in calcium deposition. The data of Table I indicate that the difference between the two control groups is large enough so that a figure midway between the results obtained with positive and negative controls would certainly be significantly different from either. The gradations in calcium deposition due to increasing amounts of test material, shown in Table II, are too small to justify statistical analysis, but the uniformity of the results obtained in the various series seems to establish the fact that vitamin D quantitatively affects the deposition of calcium under the conditions here described.

It will be noted that our basal diet is radically different from the rickets-producing diets commonly used for vitamin D studies which involve the use of the line test. None of our rats receiving only the basal diet here used developed rickets as judged by the line test (11), although occasionally a few beaded ribs were observed in rats 80 days or more in age. We were not dealing with rickets as ordinarily understood.

TABLE II.

*Effect of Graded Amounts of Supplementary Vitamin D upon Calcium Content of Femur.*

| | Supplement to basal diet per wk. | Cases. | Preexperimental period. | Experimental period. | Ca in green femur. | |
|---|---|---|---|---|---|---|
| | | | | | | Relative to controls. |
| | | | *days* | *days* | *per cent* | *per cent* |
| Series I. | None. | 12 | 0 | 35 | 7.9 | |
| | 0 75 gm. whole milk powder. | 10 | 0 | 35 | 8.0 | |
| | 1 50 " " " " | 11 | 0 | 35 | 8.9 | 37 |
| | 2 25 " " " " | 8 | 0 | 35 | 9.7 | 68 |
| | Ultra-violet irradiation.* | 11 | 0 | 35 | 10.6 | 100 |
| Series II. | None. | 6 | 31 | 28 | 7.8 | |
| | 1 50 gm. whole milk powder. | 6 | 31 | 28 | 8.9 | 32 |
| | 3 00 " " " " | 7 | 31 | 28 | 10.3 | 74 |
| | 4 50 " " " " | 9 | 31 | 28 | 11.0 | 94 |
| | Ultra-violet irradiation.* | 10 | 31 | 28 | 11 2 | 100 |
| Series III. | None. | 5 | 70–96 | 56 | 8.7 | |
| | 0.75 gm. whole milk powder. | 5 | 70–96 | 56 | 10.0 | 41 |
| | 1.50 " " " " | 7 | 70–96 | 56 | 10.8 | 66 |
| | 3.00 " " " " | 6 | 70–96 | 56 | 12.3 | 111 |
| | Ultra-violet irradiation.* | 4 | 70–96 | 56 | 11.9 | 100 |
| Series IV. | None. | 6 | 0 | 28 | 7.7 | |
| | 18 cc. fresh whole winter milk. | 6 | 0 | 28 | 8.4 | 29 |
| | 24 " " " " " | 4 | 0 | 28 | 8.7 | 42 |
| | 48 " " " " " | 4 | 0 | 28 | 9.2 | 63 |
| | 60 " " " " " | 3 | 0 | 28 | 9.5 | 75 |
| | Ultra-violet irradiation.* | 9 | 0 | 28 | 10.1 | 100 |
| Series V. | None. | 7 | 31 | 28 | 7.1 | |
| | 24 cc. fresh whole winter milk. | 7 | 31 | 28 | 8 0 | 21 |
| | 48 " " " " " | 6 | 31 | 28 | 8 5 | 33 |
| | 60 " " " " " | 3 | 31 | 28 | 9 3 | 51 |
| | Ultra-violet irradiation.* | 7 | 31 | 28 | 11.4 | 100 |

* 10 minutes daily (except Sunday) during the experimental period. The bottom of the cage was 22 inches from the arc of Alpine sun lamp No. 1111, operated on a D.C. of 110 volts; capacity 4 amperes; arc 8.0 cm.; manufactured by Hanovia Chemical and Manufacturing Company. According to Anderson (*Hanovia Chem. and Mfg. Co., Research Lab. Bull. No. 200* (1925)), lamps of this description emit an ultra-violet radiation of such a nature that in a solution containing 6.3 gm. of pure oxalic acid and 4.27 gm. of uranyl sulfate ($UO_2 \cdot SO_4 \cdot 3H_2O$) per liter, exposed in a rectangular quartz cell in a layer 1.8 cm. thick at a distance of 8 inches from the arc, the decomposition of 1 mg. of oxalic acid in 30 minutes corresponds to the absorption of 4.84 × $10^6$ ergs per sq. cm. per second.

McCollum and coworkers (12) demonstrated that the vitamin D requirement was decreased as the balance between, and the total amounts of, inorganic elements were made more nearly optimal. In common with other investigators (7, 13–16) we have observed that furnishing supplementary vitamin D increases markedly the deposition of calcium in rats receiving basal diets poor in vitamin D, even though they contain generous amounts of well balanced salt mixtures.

When the available vitamin D was limited to the store acquired before 3 to 4 weeks of age (together with possible traces found in the basal diet), the percentage of calcium in the body of our rats (negative controls) at 56 days was 0.88, at 80 days 0.98, and at 155 to 180 days 1.00. When the rats were irradiated for 10 minutes daily with ultra-violet light (positive controls), the percentage of calcium at 56 days of age was 1.02, at 80 days 1.17, and at 155 to 180 days 1.28. The basal diet used contained 0.74 per cent calcium and 0.58 per cent phosphorus ($Ca : P = 1.28$), supplied largely from a salt mixture (Osborne and Mendel) approximating the inorganic composition of milk. This being the case, the largest amount of whole milk powder fed as a supplement to the basal food did not significantly change either the percentage or the ratio of calcium and phosphorus in the total food intake. Nor did the other constituents of milk appear to play a prominent rôle in influencing calcium deposition. From 2 to 4 times the amounts of milk solids were required to effect the same changes in calcification when the milk used was low in vitamin D, *i.e.* winter milk from stall-fed cows, instead of dried whole summer milk from pasture-fed cows, which is higher in vitamin D; and a few strictly parallel experiments in which were tested corresponding amounts of natural milk powder and a synthetic milk (consisting of purified casein, lactose, a commercial hydrogenated vegetable fat, and milk ash in the proportions in which protein, fat, carbohydrate, and the inorganic elements occur in milk solids) showed that only the natural product was influential in promoting calcification in these cases.

From this experience we would offer tentatively the following method for the determination of vitamin D, when it is desired to employ a diet adequate except for vitamin D, and when the rats available as reagents possess so large a bodily store of vitamin

D as not to be readily depleted to the point where growth is inhibited. Young rats 21 to 28 days old, weighing from 25 to 45 gm., are placed on a diet like that used for vitamin A determinations, except that the constituent providing vitamin D in the vitamin A-free diet is replaced by a constituent rich in vitamin A but as nearly as practicable devoid of vitamin D. Our diet consists of extracted casein 18 per cent, Osborne and Mendel salt mixture 4 per cent, dried brewers' yeast 10 per cent, sodium chloride 1 per cent, dried powdered spinach 1 per cent, corn-starch 66 per cent. At 21 to 28 days of age they are put into individual all-metal cages with raised screen floor to prevent access to excreta, and from that time to the 56th day of age one or more of each litter of animals is kept on the basal diet without supplement; another is given an abundance of vitamin D (irradiated ergosterol in some form, or daily irradiation with ultra-violet light) and still others of the same litter are fed graded allowances of the food under investigation. The animals are kept in semidarkness in rooms lighted through glass.

At 56 days of age the rats are chloroformed, and the femurs are dissected out, carefully and quickly freed from all adhering muscle and tendons, weighed, and ashed at low red heat. The percentage of ash or of calcium in the fresh bone is determined, and taken as a criterion of the degree of calcification. In our experience, basing the percentage of ash or of calcium on the weight of the fresh femur gives results which can be quite as satisfactorily interpreted as basing results on the weight of the dried alcohol-ether-extracted bone.

We consider to have equivalent vitamin D content those amounts of materials under investigation which induce a degree of calcification midway between the minimum values fixed by the diet without added vitamin D and the maximum values obtained with an abundance of supplementary vitamin D. It should be emphasized that this method is feasible only when the groups of animals used for testing vitamin D can be compared with two control groups each containing representatives from the same litters and matched in sex and weight, one receiving no added vitamin D, the other receiving a fixed liberal supply. Probably animals from at least five or more litters should be used for obtaining the average figures for each group.

## SUMMARY AND CONCLUSIONS.

1. In using rats such as ours, which have considerable bodily stores both of vitamins A and D, for quantitatively determining these vitamins under conditions in which the vitamin in question is the first dietary deficiency, we find gain in weight under suitably controlled conditions the best measure for vitamin A intake, and the degree of calcification the most practical measurement of vitamin D intake (in test animals which do not show rickets).

2. The experience of our laboratory indicates clearly that quantitative comparisons of the vitamin A content of materials are best made by determining the amounts necessary to produce the same limited gain in weight (3 or 4 gm. per week during the test period) in a sufficient number of suitably standardized rats receiving a basal diet free from vitamin A, but otherwise adequate.

3. Less extensive but quite consistent experience indicates that quantitative comparisons of the vitamin D content of food (or other) materials may be made by finding the amounts necessary to induce a degree of calcification midway between those maximum and minimum values obtainable under suitably controlled conditions, although the actual percentage of calcium in the animal will depend on the nutritional history, its initial age, weight, and sex, as well as on the mineral constituents of the basal diet, and the length of the experimental period.

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# STUDIES ON THE PHYSIOLOGY OF PYRIMIDINES.

## III. THE INTERMEDIARY METABOLISM OF URACIL.*

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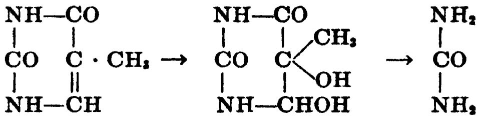
(Received for publication, May 7, 1930).

Some time ago, the writer (1) studied the metabolism of certain pyrimidines. That investigation showed that thymine glycol which is an oxidation product of thymine *in vitro*, is oxidized in the dog to the same end-products as thymine. On the basis of those observations it was assumed that the oxidation of thymine in the animal body takes place according to the reaction:

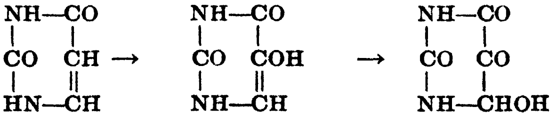


Thymine. Thymine glycol. Urea.

The fate of the remainder of the thymine molecule was not determined.

Those results led to the investigation of the intermediary metabolism of uracil which is the subject of the present paper. Baudisch (2) has shown that isobarbituric acid is an intermediate product in the oxidation of uracil *in vitro*. Behrend and Roosen (3) found that isobarbituric acid is converted by bromine water into isodialuric acid.

We are dealing with the following reaction.



Uracil. Isobarbituric acid. Isodialuric acid.

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The present investigation was undertaken in an attempt to determine whether isobarbituric acid and isodialuric acid are intermediary products in the metabolism of uracil in the animal body. Steudel (4) studied the behavior of these compounds in a dog. He found that they were apparently completely oxidized since he was not able to recover them from the urine collected following their administration.

## EXPERIMENTAL.

Isobarbituric acid was prepared according to the method of Davidson and Baudisch (5). Isodialuric acid was obtained from isobarbituric acid on oxidation with bromine water (3).

TABLE I.

*Experiments with Isobarbituric Acid.*

Dog A; weight 11 kilos.

| Day. | Experiment 1. | | | | | Experiment 2. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Volume. | Total N. | Urea N. | Ammonia N. | Inorganic S. | Volume. | Total N. | Urea N. | Ammonia N. | Inorganic S. |
| | *cc.* | *gm.* | *gm.* | *gm.* | *gm.* | *cc.* | *gm.* | *gm.* | *gm.* | *gm.* |
| 1 | 225 | 5.75 | 4.56 | 0.46 | 0.276 | 183 | 4.86 | 4.01 | 0.56 | 0.209 |
| 2 | 211 | 5.59 | 4.51 | 0.43 | 0.259 | 187 | 4.88 | 3.99 | 0.55 | 0.214 |
| 3 | 205 | 5.60 | 4.62 | 0.44 | 0.268* | 175 | 5.01 | 4.13 | 0.47 | 0.209* |
| 4 | 219 | 5.97 | 4.83 | 0.37 | 0.138 | 183 | 5.49 | 4.36 | 0.41 | 0.043 |
| 5 | 189 | 5.49 | 4.54 | 0.42 | 0.236 | 180 | 5.02 | 4.19 | 0.52 | 0.190 |
| 6 | 192 | 5.57 | 4.55 | 0.33 | 0.259 | 164 | 5.04 | 4.20 | 0.51 | 0.210 |

* 3.0 gm. of isobarbituric acid fed with food. N = 0.66 gm.

Female dogs were used exclusively in the experiments described below. The animals were kept on the standard diet described by Cowgill (6). The plan of the experiments was to follow the various urinary constituents until a nitrogen equilibrium had been reached, to give the substance to be tested mixed with the food, then to continue the experiment until the output of these excretory products had returned to normal. The dogs were kept in metabolism cages, and the urine collected by catheterization every 24 hours. The animals were allowed to drink water *ad libitum*.

The following methods for analyses were used: total N, Kjeldahl; urea, Van Slyke's gasometric method; ammonia, Folin's permutit method; inorganic sulfur, precipitation with benzidine and titration according to Drummond and Folin's gravimetric method; total and ethereal sulfates, Folin's method; total sulfur, Givens' modification of Benedict's method (7).

### *Experiments with Isobarbituric Acid.*

The results obtained after feeding isobarbituric acid are shown in Table I. An examination of the data shows that the substance was to a great extent broken down to urea. Attention is called to the figures showing the output of inorganic sulfates. Following the feeding of the compound we find a distinct drop in the excretion of inorganic sulfates.

TABLE II.

*Experiments with Isodialuric Acid.*

| | Day. | Volume. | Total N. | Urea N. | Ammonia N. | Inorganic S. |
|---|---|---|---|---|---|---|
| | | *cc.* | *gm.* | *gm.* | *gm.* | *gm.* |
| Dog A; weight 11.2 kilos. | 1 | 200 | 5.31 | 4.27 | 0.48 | 0.252 |
| | 2 | 185 | 5.29 | 4.35 | 0.42 | 0.256 |
| | 3 | 190 | 5.27 | 4.40 | 0.41 | 0.234* |
| | 4 | 195 | 5.73 | 4.76 | 0.49 | 0.168 |
| | 5 | 200 | 5.48 | 4.61 | 0.42 | 0.281 |
| | 6 | 215 | 5.35 | 4.41 | 0.44 | 0.237 |
| Dog B; weight 9.5 kilos. | 1 | 140 | 4.57 | 4.13 | 0.26 | 0.223 |
| | 2 | 104 | 4.69 | 3.99 | 0.29 | 0.215 |
| | 3 | 100 | 4.69 | 4.03 | 0.31 | 0.217* |
| | 4 | 150 | 5.46 | 4.31 | 0.31 | 0.181 |
| | 5 | 180 | 4.95 | 4.10 | 0.28 | 0.230 |
| | 6 | 115 | 4.72 | 4.15 | 0.27 | 0.212 |
| Dog C; weight 12.0 kilos. | 1 | 146 | 5.65 | 4.36 | 0.49 | 0.287 |
| | 2 | 151 | 5.59 | 4.48 | 0.43 | 0.281 |
| | 3 | 178 | 5.67 | 4.52 | 0.40 | 0.292* |
| | 4 | 195 | 6.26 | 5.08 | 0.31 | 0.241 |
| | 5 | 157 | 5.79 | 4.67 | 0.42 | 0.294 |
| | 6 | 140 | 5.71 | 4.55 | 0.44 | 0.291 |

* 3.0 gm. of isodialuric acid fed. N = 0.58 gm.

### *Experiments with Isodialuric Acid.*

The data from three experiments on three different dogs illustrating the metabolism of isodialuric acid are found in Table II.

In every case a rise in the urea output was noted after feeding this substance. Coincident with this rise in urea we find also in these experiments a marked decrease in the excretion of inorganic sulfates.

### *Effects of Isobarbituric Acid and Isodialuric Acid on Sulfur Metabolism.*

As was indicated above, isobarbituric acid and isodialuric acid caused a distinct drop in the excretion of inorganic sulfates. These observations raised the question as to what effect the ingestion of these substances might have on the other fractions of urinary sulfur. Two experiments (Table III) bring the answer to that question. An examination of Table III shows that following the feeding of isobarbituric acid, there is, coincident with the drop of inorganic sulfates, a proportionate increase in the output of ethereal sulfates. Inasmuch as it was found that the feeding of isodialuric acid also affected the output of inorganic sulfur in the

TABLE III.

*Observations on Sulfur Metabolism.*

| | Day. | Volume. | Total N. | Urea N. | Ammonia N. | Inorganic S. | Total S. | Total sulfate S. | Ethereal S. | Neutral S. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *cc.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| Dog B; | 1 | 111 | 5 44 | 4 71 | 0.34 | 0.220 | | | | |
| weight 9.6 | 2 | 113 | 5.34 | 4.55 | 0.26 | 0 210 | | | | |
| kilos. | 3 | 140 | 5 28 | 4 56 | 0.33 | 0.216 | 0.297 | 0.240 | 0.024 | 0 057* |
| | 4 | 105 | 5 76 | 4.81 | 0.28 | 0.084 | 0 288 | 0 237 | 0.153 | 0.051 |
| | 5 | 95 | 5.15 | 4.32 | 0.27 | 0.159 | 0.236 | 0.188 | 0.029 | 0 048 |
| | 6 | 115 | 5.14 | 4.42 | 0.26 | 0.210 | 0 283 | 0.233 | 0.023 | 0.050 |
| Dog C; | 1 | 159 | 5.58 | 4.44 | 0.43 | 0.242 | | | | |
| weight | 2 | 147 | 5.41 | 4.30 | 0.44 | 0.216 | | | | |
| 12.0 kilos. | 3 | 149 | 5.50 | 4.50 | 0.44 | 0.220 | 0.316 | 0.242 | 0.022 | 0.074* |
| | 4 | 155 | 5.97 | 4.80 | 0.39 | 0.089 | 0.285 | 0.238 | 0.149 | 0.047 |
| | 5 | 143 | 5 32 | 4.21 | 0.45 | 0.197 | 0.278 | 0.228 | 0.031 | 0.050 |
| | 6 | 138 | 5.60 | 4.46 | 0.51 | 0.247 | 0.337 | 0.275 | 0.028 | 0.062 |
| | 7 | 147 | 5 61 | 4.52 | 0.43 | 0.223 | | | | |

* 2.5 gm. of isobarbituric acid fed. N = 0 56 gm.

same direction as isobarbituric acid, it is reasonable to assume that these two substances are to a greater extent broken down to urea and to a lesser extent eliminated as ethereal sulfates. The latter part of this assumption has to remain tentative until it is possible to isolate the isobarbituric acid and isodialuric acid sulfates from the urine.

## DISCUSSION.

The observations recorded in the present series of experiments seem to indicate that isobarbituric acid and isodialuric acid are intermediary products in the metabolism of uracil. Apparently, we are dealing with the following sequence of reactions: uracil→isobarbituric acid→isodialuric acid→urea and a carbon compound containing 3 carbon atoms.

It is significant that, as in the case of thymine, we are dealing here with oxidations taking place in animal tissues which are similar to those occurring *in vitro.*

Another important fact which emanates from these experiments is the distinct drop in the output of inorganic sulfur and the proportionate rise in the excretion of ethereal sulfates following the feeding of these compounds. It appears reasonable to assume that isobarbituric acid and isodialuric acid are partly excreted as ethereal sulfates. It seems to the writer that these observations will make it necessary to devote especial attention to the different fractions of sulfur in the urine in experiments dealing with the metabolism of purines and pyrimidines.

## SUMMARY.

Experiments are described in which isobarbituric acid and isodialuric acid were fed to dogs maintained on a nitrogenous equilibrium.

Evidence was obtained that isobarbituric acid and isodialuric acid, when fed in small amounts to dogs, are to a great extent metabolized to yield urea.

It is assumed that in the metabolism of uracil we are dealing with the following sequence of reactions: uracil→isobarbituric acid→isodialuric acid→urea + an unknown carbon compound.

Following the feeding of isobarbituric acid and isodialuric acid a distinct drop in the output of inorganic sulfates was noted.

This drop in the excretion of inorganic sulfates is due to a practically proportionate increase in ethereal súlfate. Pending the isolation of isobarbituric acid and isodialuric acid sulfate from the urine it is tentatively assumed that these compounds are excreted partly in the form of ethereal sulfates.

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# THE $\alpha$-OXIDATION OF ACETALDEHYDE AND THE MECHANISM OF THE OXIDATION OF LACTIC ACID.

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In previous papers (1, 2) it has been shown that in dilute aqueous solutions aldehydes may be oxidized in the $\alpha$ position by oxidizing agents such as ceric sulfate, potassium dichromate, and potassium ferricyanide. Normal butyraldehyde and isobutyraldehyde yielded the most conclusive results; the latter was converted by ceric sulfate at 80° into $\alpha$-hydroxyisobutyraldehyde and acetone. The evolution of carbon dioxide by the oxidation of acetaldehyde with potassium permanganate in acid solution under special conditions indicated that the $\alpha$-oxidation could proceed also with this aldehyde. A study of the action of ceric sulfate on acetaldehyde in dilute solution at 80° has now shown that with this reagent $\alpha$-oxidation is the normal course of the reaction. A study of the action of the same reagent on lactic acid shows that the acetaldehyde first formed undergoes $\alpha$-oxidation. The possible biochemical implications of this fact are considered in the concluding paragraphs of this paper.

## *Oxidation of Acetaldehyde.*

The oxidation of acetaldehyde by a 0.2 N solution of ceric sulfate in 0.5 M sulfuric acid at 80° yields formic acid as the chief product. In addition, about 0.25 mol of carbon dioxide per mol of aldehyde is produced and small amounts of volatile and non-volatile aldehydes. The presence of glycolic aldehyde or glyoxal was established by the formation of the *p*-nitrophenylosazone which was identified by a mixed melting point. The fact that the solution (after removal of the cerous salts) reduced Fehling's solution rapidly at room temperature but reacted very slowly with phenylhydrazine showed that the source of the osazone was glycolic aldehyde and not glyoxal, although the presence of some of this compound is not excluded. Glyoxylic acid is also formed in varying amounts depending on the conditions. A positive test for it was obtained in the distillate in several experiments. A small amount of formaldehyde is probably also formed but not in sufficient amounts to obtain a solid derivative.

In all the experiments the temperature was kept at 80–90°; in order to study the volatile aldehyde it was necessary to be sure that all the acetaldehyde had been oxidized. This was accomplished in a number of experiments by employing a 5:1 molecular proportion of oxidizing agent and prolonging the action for 2 hours. A typical experiment was as follows: 50 cc. of freshly prepared 0.2 M acetaldehyde solution (0.01 of a mol) were added to 200 cc. of a 0.24 M solution of ceric sulfate (0.048 of a mol) in 0.5 M sulfuric acid. The temperature was kept at 80–90° for 2 hours; a slight excess of ceric sulfate was still present at the end of this time, as shown by the yellow color. The solution was cooled, filtered (a large amount of cerous sulfate precipitated during the reaction), and about 200 cc. distilled. The distillate contained no acetaldehyde as judged by the absence of a positive iodoform test. Tests with fuchsin reagent, and Tollens' reagent, were positive, while the solution failed to reduce Fehling's solution. A slight murkiness was formed in treating the distillate with *p*-nitrophenylhydrazine. A Hopkins-Cole test for glyoxylic acid was positive. Another sample of the distillate was neutralized with sodium hydroxide (in some experiments a redistillation was performed before the neutralization). The sodium salt was obtained by evaporation to dryness (each run yielded a few tenths of a gm.). The salt was found to be chiefly sodium formate contaminated with a little sodium carbonate. (Possibly small amounts of sodium acetate were also present.) The typical reactions of sodium formate (including the evolution of 1 mol of carbon dioxide on oxidation), an analysis of the barium salt, and the actual isolation of formic acid from several gm. of material, completed the identification.

The nature of the non-volatile aldehyde was established in another similar experiment in which the reaction mixture stood 15 minutes and only 150 cc. were distilled in order to avoid decomposition. Such a residue on treatment with *p*-nitrophenylhydrazine yielded a small amount of reddish brown precipitate (a few mg. in each experiment). This precipitate gave the test with sodium hydroxide and alcohol characteristic of the *p*-nitrophenylosazone of glyoxal. The yield of the osazone could be increased by evaporation of the initial solution to a small volume before the precipitation; the evaporation was carried out under diminished pressure at 80°. After almost one-third of the water had been distilled, the solution was neutralized with sodium carbonate, the precipitated cerous carbonate filtered off, and the evaporation continued, inorganic solids being removed from time to time. In this way the volume was diminished to almost 0.1 of the original. The concentrated solution reduced Fehling's solution in the cold; in the Hopkins-Cole test for glyoxylic acid a deep brown ring was formed which obscured the glyoxylic acid reaction if such were present. About 1 gm. of *p*-nitrophenylosazone per 0.05 mol of acetaldehyde was obtained from this concentrated solution; this is a 10 per cent yield if the precipitate is assumed to be pure glycolic aldehyde osazone. The orange color and the fact that a portion of it was soluble in alcohol indicated that the *p*-nitrophenylhydrazone of glyoxylic acid was also present. Pure *p*-nitrophenylosazone of glycolic aldehyde was obtained by extraction with alcohol and recrystallization from pyridine; identification was made complete by a mixed melting point with a known specimen. Experiments in which the molecular ratio of ceric sulfate to aldehyde was 2:1 and 4:1 yielded the same amount of product.

### *Oxidation of Lactic Acid.*

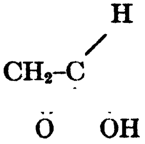
If a solution of lactic acid and a solution of 0.2 M ceric sulfate are slowly dropped into boiling 0.5 M sulfuric acid, the distillate contains acetaldehyde (identified by the formation of the *p*-nitrophenylhydrazone). However, if the acetaldehyde is not removed immediately but allowed to react further with the ceric sulfate, the final products of the oxidation of lactic acid by ceric sulfate are glycolic aldehyde, glyoxylic acid, formic acid, and formaldehyde. Strangely enough the yield of formaldehyde is greater than when acetaldehyde itself is oxidized.

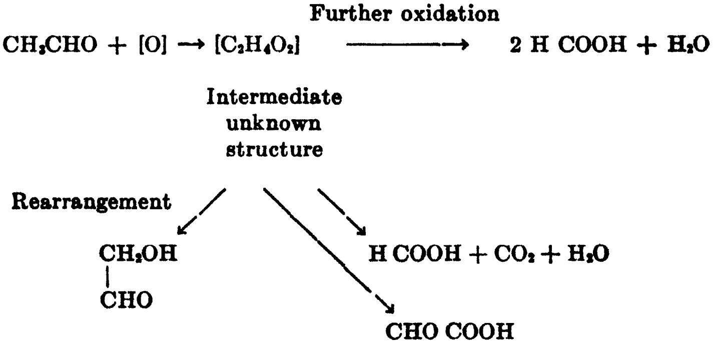
A typical experiment was as follows: To 200 cc. of 0.24 M ceric sulfate (0.048 mol) in 0.5 M sulfuric acid at 80°, 35 cc. of 0.2 M lactic acid (0.007 mol) were added either at once or slowly. (In some experiments a solution of recrystallized zinc lactate was employed without altering the results.) After 2 hours, the solution was distilled (a slight excess of ceric sulfate was present) and the distillate examined in exactly the same manner as described in the experiments with acetaldehyde. The fuchsin and Tollen's tests were very much stronger than in the experiments with acetaldehyde while the glyoxylic acid test was of about the same intensity; the iodoform test was negative. The *p*-nitrophenylhydrazone of formaldehyde was obtained as a yellow solid by the addition of the reagent to the distillate. It was identified by a mixed melting point determination. The chief component of the distillate was identified as formic acid.

The essentially non-volatile products of the oxidation of lactic acid by ceric sulfate are a mixture of glyoxylic acid and glycolic aldehyde as in the case of acetaldehyde. As a rule the yield of these products seems to be less; glyoxylic acid appears to be the sole product when a large excess of ceric sulfate is employed. Thus, if lactic acid is slowly added to boiling ceric sulfate in a 6:1 molecular ratio, only half of the ceric sulfate is reduced since the major portion of the product is acetaldehyde which at once escapes. After concentration of the residual liquid, a yellow precipitate was formed with *p*-nitrophenylhydrazine which had the same appearance and solubility as the *p*-nitrophenylhydrazone of glyoxylic acid (a complete identification was not possible because of the unsatisfactory melting point of this compound); the Hopkins-Cole test was strongly positive. The absence of glycolic aldehyde was shown by the fact that the solution did not reduce Fehling's solution at room temperature and by the yellow color and solubility of the precipitate with *p*-nitrophenylhydrazine. It being assumed that the yellow precipitate was composed solely of the hydrazone of glyoxylic acid, the yield was about 3 per cent.

The best yield of glycolic aldehyde from lactic acid was obtained by having only a 4:1 ratio of ceric sulfate to acid and allowing the reaction mixture to stand 15 to 30 minutes at 80° before distillation. The concentrated solution obtained under these conditions reduced Fehling's solution in the cold, gave a brown ring with no purple color in the Hopkins-Cole test, and yielded an orange precipitate with *p*-nitrophenylhydrazine. The pure *p*-nitrophenylosazone was readily prepared from this precipitate by the procedure described above. It was identified by a mixed melting point determination. The yield was about 8 per cent. With a ratio of 6:1 of reagent to acid, the yield was only about 3 per cent and though the precipitate was orange the preponderance of glyoxylic acid was shown by a strong Hopkins-Cole test and a negative Fehling's test in the cold.

DISCUSSION.

That the first step in the oxidation of acetaldehyde by ceric sulfate consists in the introduction of oxygen in the $\alpha$ position can hardly be doubted in view of the facts given above. The possibility of the process proceeding through acetic acid seems excluded by the fact that acetic acid, like formic acid, is not attacked by hot ceric sulfate. We cannot be certain that the intermediate product between acetaldehyde and formic acid is glycolic aldehyde. Some unstable intermediates such as  $\underset{\ddot{O}}{CH_2}-\underset{OH}{\overset{H}{C}}$ may first be formed, but the formation of the $p$-nitrophenylosazone of glyoxal from the reaction mixture proves that some glycolic aldehyde is present at the end. The formation of formic acid may proceed through glycolic acid but this substance on oxidation with ceric sulfate yields 1 mol of carbon dioxide for each mol of formic acid, whereas the ratio of formic acid to carbon dioxide with acetaldehyde is about 3:1. The most conservative method of formulating our results would seem to be as follows:


Further oxidation
CH₃CHO + [O] → [C₂H₄O₂] → 2 H COOH + H₂O
Intermediate unknown structure
Rearrangement
CH₂OH | CHO
H COOH + CO₂ + H₂O
CHO COOH

Heimrod and Levene (3) found that the oxidation of acetaldehyde by hydrogen peroxide in alkaline solution yielded formic acid. They assumed that the intermediates were vinyl alcohol, glycolic aldehyde, and glyoxal, and showed that the last two substances yielded formic acid under the conditions of the experiment. Our identification of glycolic aldehyde and glyoxylic acid among the products of the oxidation of acetaldehyde might be taken as supporting evidence for their hypothesis although our reagent and conditions were very different from theirs. The enol form of acetaldehyde may be involved in oxidations which occur in alkaline solution (Heimrod and Levene's least alkaline conditions were with dilute ammonia), but it is much less probable that a reaction would take this course in 20.5 M sulfuric acid. Furthermore, the oxidation of vinyl alcohol directly to glycolic aldehyde by the oxidation of two hydroxyl groups to the ethylene linkage is very improbable since ceric sulfate does not attack the double carbon linkage. It differs markedly in this respect from the equally strong oxidizing agent, potassium permanganate, and from hydrogen peroxide. For this reason we prefer to write an intermediate $C_2H_4O_2$ of unknown structure. Considering the fact that the yield of glycolic aldehyde is about the same whether a 2:1 or a 4:1 ratio of oxidizing agent is employed, it seems as if this aldehyde were produced by a side reaction.

We must conclude that acetaldehyde is an intermediate in the main reaction between ceric sulfate and lactic acid since it escapes from the reaction mixture if the opportunity is provided. The further oxidation of acetaldehyde then yields the products noted in the scheme outlined in the preceding paragraph. This simple explanation is not entirely satisfactory, however, since it does not account for the fact that very definite amounts of formaldehyde are formed from lactic acid while with acetaldehyde the amount of this aldehyde in the oxidation products was too small to make its identification certain. Probably in addition to the main reaction which follows the course lactic acid → acetaldehyde, there is another side reaction in which lactic acid is oxidized to formaldehyde. The other products of this side reaction may well be identical with those formed from acetaldehyde. Another possibility is that the first oxidation product of acetaldehyde may react with lactic acid, one of the products of this interaction being formaldehyde.

The results obtained with lactic acid and acetaldehyde suggest that perhaps the same mode of oxidation may occur in biological processes. One would expect that under certain circumstances the first product of the oxidation of acetaldehyde (*i.e.*, glycolic aldehyde or the intermediate $C_2H_4O_2$) might undergo reactions other than those we have found. In particular, one would expect polymerization to carbohydrates. The mechanism of the oxidation of lactic acid in the muscle and the resynthesis of glycogen might be imagined to take some such course as the following.

$$3\ CH_3CHOH\ COOH + 3\ O_2 \rightarrow 3\ [C_2H_4O_2] + 3\ CO_2 + 3\ H_2O$$

$\downarrow$

Intermediate

$\downarrow$

Polymerization

Glycogen ($C_6H_{10}O_5$) + $H_2O$

The actual process in the muscle is specific for lactic acid (and pyruvic) and impossible of duplication with acetaldehyde or glycolic aldehyde. This is not inconsistent with the suggested mechanism; such a situation appears to exist in regard to the production of formaldehyde by oxidation *in vitro*, as has been shown above. The ratio of oxygen to carbon dioxide given by the above equation is, of course, in accord with the facts. The portion of acid resynthesized is two-thirds (0.66) according to the scheme outlined above, while Meyerhof (4) believes that under favorable circumstances almost four-fifths (0.80) of the lactic acid may be resynthesized. If one were willing to postulate that a molecule of lactic acid or its isomer methyl glyoxal as well as the intermediate were involved in the polymerization, the following reaction involving the resynthesis of four-fifths of the acid might be written:

$$3\ C_2H_4O_2 + 2\ C_3H_6O_3 \rightarrow 2\ C_6H_{10}O_5 + 2\ H_2O$$

While this mechanism has the advantage of clearly explaining why the process is specific for lactic acid, it would involve the cleavage of 1 molecule of the compound $C_2H_4O_2$ into formaldehyde in order for the six carbon chain in glycogen to be synthesized. These mechanisms provide a direct chemical path by which the energy of the oxidation process might effect the resynthesis of the carbohydrate. Since it has been shown that the oxidation *in vitro* of lactic acid yields such reactive substances as formaldehyde and glycolic aldehyde, it is very tempting to imagine that similar products are formed in the muscle where they would be just the compounds needed for the resynthesis of carbohydrates without further expenditure of energy.

We are in indebted to Mr. W. F. Bruce for valuable assistance in performing some of the experiments reported in this paper.

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# ANTIRICKETIC SUBSTANCES.

## X. ON THE RELATION OF THE ISOERGOSTEROLS TO VITAMIN D.

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In 1928, Bills, Honeywell, and Cox (1) offered evidence that the absorption band, λ 248 *mμ*, exhibited by some samples of irradiated ergosterol, is due to a degradation product of vitamin D. Attention was called to the striking similarity of this band and the absorption band of isoergosterol, indicating a similarity in molecular configuration of the two absorbing substances (*cf.* van Wijk and Reerink (2)). Several experiments during the past year have emphasized the closeness of this relationship. For instance, we have observed that when solutions of isoergosterol are irradiated, the band fades like the band of the degradation product. Moreover, the *rates of fading* of the two are similar. And again, the fading of each is similarly accelerated by the presence of air in the quartz cell.

Bills and McDonald have recently demonstrated (3) that the several isoergosterols which result from the action of acid catalysts on ergosterol are complex mixtures, only partially separable by many crystallizations. The components apparently possess in common the absorption band at 248 *mμ*, and, as ordinarily prepared, the mixtures exhibit a molecular extinction coefficient of about 16,000. We may therefore speak loosely of isoergosterol as a substance characterized by this band and extinction coefficient.

In comparing the vitamin degradation product with isoergosterol, it is desirable to determine not only the position of the absorption maximum, but also its height. Since the degradation product has not yet been isolated, its extinction coefficient can be ascertained only indirectly. It occurred to us that if the two substances are identical or very similar, it should be possible to duplicate the absorption curve of overirradiated ergosterol with a mixture of ergosterol and isoergosterol in suitable proportion.

In Fig. 1, *A* we show the absorption curves of a typical ergosterol, $[\alpha]^{25}_{5461} = -157°$, and of a typical isoergosterol, $[\alpha]^{25}_{5461} = -52°$ (HCl catalyst). The curves were made with a Hilger quartz spectrograph, sector photometer, and water-cooled discharge tube.

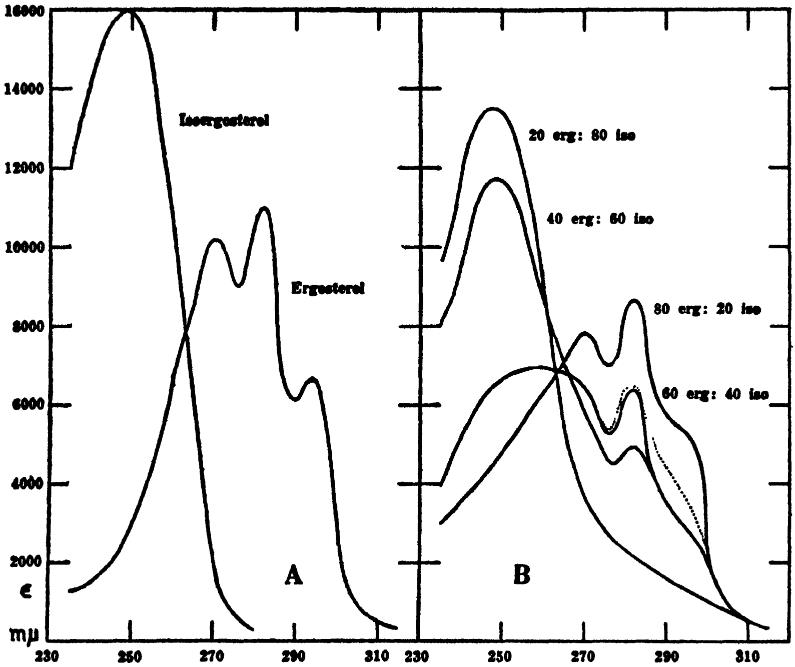

FIG. 1. Ultra-violet absorption of (*A*) ergosterol and isoergosterol, (*B*) mixtures of ergosterol and isoergosterol in comparison with vitamin degradation product.

The tube contained hydrogen at 4 mm. of Hg pressure, and was operated by a 1 kilowatt, 25,000 volt transformer in parallel with a condenser of 0.009 microfarad capacity. Such a light source gives with pure, dry hydrogen a continuous ultra-violet spectrum particularly suitable for absorption spectroscopy.

In Fig. 1, *B* we show the absorption curves of ergosterol-isoergosterol mixtures in the ratios of 20:80, 40:60, 60:40, and

80:20. In comparison with these is the dotted curve of a slightly overirradiated ergosterol. The latter preparation had been exposed in alcohol for 45 minutes to a water-jacketed mercury arc, under the conditions described in our previous paper (1). The peak of potency had come at 22.5 minutes, the solute being 250,000 times as antiricketic as average cod liver oil. By 45 minutes the potency had declined to 140,000 ×. The greatest activation that we have ever attained (under special conditions) was about 1,000,000 ×, but even this product was not pure vitamin D. Hence the absorption curve of the 45 minute irradiation product does not reflect any large error from the presence of vitamin D, provided, of course, that the vitamin itself does not exhibit an exceptionally powerful absorption.

Parenthetically, it is to be noted that attempts to determine the absorption curve of vitamin D have not given concordant results. Bills, Honeywell, and Cox (1) concluded that: "The wave-lengths which vitamin D itself absorbs, and by which it is destroyed, apparently lie within the same spectral region as the wave-lengths which activate ergosterol." Smakula (4) interpreted a study of difference curves to indicate that vitamin D is characterized by two bands in this region, $\lambda$ 293 and 262 $m\mu$. Bourdillon, Fischmann, Jenkins, and Webster (5) associated vitamin D with a single band of great intensity, $\lambda$ 280 $m\mu$. Reerink and van Wijk (6) calculated for vitamin D a single broad band, with a maximum near 268 $m\mu$. Windaus has recently reported (7) that certain impure preparations of vitamin D show an absorption maximum at 270 $m\mu$. Particularly significant is the work of Jendrassik and Keményffi (8), who have succeeded in preparing a highly potent activated ergosterol that showed almost no absorption. Our early work tends to support theirs, for in the parallel series of spectrographic and quantitative biologic measurements we found nothing but a diminution of absorption until the peak of potency was reached. Then the band at 270 $m\mu$ broadened and began to shift, in response to the increasing amount of degradation products. If it be true that pure vitamin D is transparent, it follows that its disappearance upon prolonged irradiation is not directly photochemical.

From the investigations cited above, it is known that during the irradiation of ergosterol, not merely one degradation product, but a succession of them, is produced. Under the conditions of our experiment the substance, λ 248 *mμ*, is one of the earliest. It is more stable than vitamin D, but eventually it is replaced by still other forms. That these later forms were not present to a noteworthy extent is shown by the following: The shortest period of irradiation sufficient to produce a spectrographically detectable amount of the early degradation product was 45 minutes. 3 hours were required to produce it in maximum concentration. Even 15 hours did not destroy all evidence of absorption at 248 *mμ*. It is a reasonable conclusion that the solution selected for comparison with the known mixtures consisted mainly of ergosterol and the early degradation product of vitamin D.

It is well known that with mixtures of substances having overlapping absorption bands, determinations of the position and height of the bands are subject to error. For example, in the curve of ergosterol-isoergosterol 60:40, the isoergosterol has come under the influence of the shorter wave-lengths of the ergosterol absorption, and its absorption curve is abnormal laterally, although not much affected vertically. On the other hand, the principal ergosterol band is not affected by the comparatively distant isoergosterol absorption, and consequently its maximum lies as usual at 282 *mμ*, and its extinction coefficient is almost exactly 60 per cent of that of ergosterol.

The dotted curve in Fig. 1, *B* is almost a duplicate of the curve of the 60:40 ergosterol-isoergosterol mixture. The absorption curve of the early degradation product is therefore the same, not only in position, but also in height, as that of isoergosterol, λ 248 *mμ*, ε 16,000.

Unfortunate for complete identification is the fact that the early degradation product, unlike the known isoergosterols, does not give a precipitate with digitonin. This may indicate merely that the digitonide, if formed, is soluble. Windaus and Auhagen (9) have shown that certain sterols do not precipitate with digitonin. On the other hand, this non-precipitability may be the result of a change in the alcohol group of the sterol—a group which is known to exert but little influence on sterol absorption bands, but which is concerned in the formation of complexes with digitonin.

Our early degradation product is probably the "substance B" of Bourdillon, Fischmann, Jenkins, and Webster (5), although these authors place its absorption maximum at 240 $m\mu$. Rosenheim and Adam (10), on the basis of spectrographic comparisons and monomolecular film measurements, are inclined to associate "substance B" and the vitamin with the sterol ketones. Our findings indicate equally well a relationship with the isoergosterols.

## SUMMARY.

1. An alcoholic solution of ergosterol was exposed to the radiations of a quartz mercury lamp until the antiricketic potency that developed had begun to decline. In the solution there was found a substance, probably a degradation product of vitamin D, which exhibited an absorption spectrum characteristic of the isoergosterols, $\lambda$ 248 $m\mu$, $\epsilon$ 16,000.

2. This substance is distinguished from the ordinary isoergosterols by the fact that it, like vitamin D, does not form a precipitate with digitonin.

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# DIFFUSIBLE NON-PROTEIN CONSTITUENTS OF BLOOD AND THEIR DISTRIBUTION BETWEEN PLASMA AND CORPUSCLES.

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The transformation of the Folin-Wu method for the preparation of protein-free blood filtrates into a method giving protein-free extracts from unlaked blood (1) involves experimentally only the simplest sort of modification, but produces results of far reaching consequences. There will be necessarily a sharp break between the hospital records or the past published records of blood analyses based on aqueous extracts from laked whole blood and corresponding records obtained from unlaked blood. But the conclusion seems inescapable that for nearly every kind of metabolism study, it must be obscuring and misleading to include in the analyses of blood as a transportation system, an unknown mixture of residues obtained from the destroyed cellular elements which have no direct connection with the circulating waste products or food products.

The disadvantage arising from a loss of continuity between past records and new ones made on unlaked blood should be partly compensated for by the fact that in the future there should be less need for revisions of methods. In the field of sugar determinations, for example, we now find that the Folin-Wu and Folin methods (1929 revisions) give practically identical values both with normal and with diabetic bloods. So far as can now be seen, the only copper method for blood sugar to be used hereafter in this laboratory will be the 1929 revision of the Folin-Wu method (2), because all our blood analyses will be based on unlaked blood.[1] The Folin method will become superfluous.

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[1] S. R. Benedict has privately pointed out to one of us that the copper reagent of the 1929 method does not have exactly the same composition as

Our primary object in beginning the work described in this paper was to supply analytical data on laked and unlaked blood in order to show just how the two extracts differ. But this comparison soon became a side issue, because another comparison, namely that between unlaked blood and plasma, became so much more instructive.

*Distribution of Diffusible Products between Plasma and Corpuscles.*—In the course of our comparative analyses of protein-free extracts from unlaked blood and plasma, it became apparent that the distribution of the diffusible products between plasma and corpuscles must be represented in the literature by decidedly erroneous figures, and that the true distribution, in fact, scarcely could be determined by the methods heretofore used for its investigation.

*1. Sugar.*—The concensus of opinion has been that blood sugar in man tends to be equally distributed between plasma and corpuscles. This conclusion has been so well supported by work from different laboratories that for the past 5 years or more it has scarcely been questioned. But this conclusion is, nevertheless, wrong. The concentration of glucose in the corpuscles is very much lower than in the plasma, and this fact is one of the reasons why any good method will yield relatively low blood sugar values when applied to extracts from unlaked blood. That the blood sugar cannot be equally distributed between cells and plasma becomes more or less self evident from the fact that nearly the whole of the non-fermentable material responsible for the residual reduction of laked blood extracts is found in the corpuscles. The unequal distribution could have been demonstrated by direct total reduction determinations by the use of the more selective copper reagents, such as the weakly alkaline reagent described by Folin, but it has just happened that no one has reinvestigated the distribution problem since the more selective sugar methods were found.

While this work was in progress, there appeared a paper by Ege

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in the original method, and that it might be more or less confusing or even misleading to refer to it as the Folin-Wu reagent. The 1929 method represents the last of a series of revisions, and referring to it as the Folin-Wu method of 1929 would seem to supply a concise and historically accurate description.

and Roche (3), in which Ege modifies his earlier view about the distribution of the blood sugar between plasma and corpuscles. He now comes to the plausible conclusion that the distribution of the blood sugar should be equal in the water of the cells and plasma, and that actually, therefore, the concentration of the sugar in the cells should be about 80 per cent of the concentration found in the plasma, since the cells contain only about 0.8 times as much water as does the plasma.

The Danish paper contains many analytical data on the distribution of the blood sugar, but there is seemingly no connection or correspondence between these analyses and Ege's theoretical concept. The paper contains no comments on the discrepancy. One of the analytical methods used by Ege and Roche, namely the micro method of Fontès and Thivolle, has given values which in fact are quite impossible. In six out of eight blood samples examined, the concentration of the fermentable sugar as found by this method is actually greater in the corpuscles than in the plasma. These bizarre results prove, of course, only that the Fontès-Thivolle sugar method is hopelessly unusable. The method is based on the indefensible assumption that only insoluble cuprous oxide furnishes a true measure of reduction due to sugar, thus quite ignoring the fact that blood filtrates contain materials which, like ammonia, are capable of preventing the precipitation of cuprous oxide.

When working as we now do with unlaked blood it is not necessary to make separate determinations of residual reduction in order to show that the blood sugar is not equally distributed between plasma and corpuscles. Moreover, the distribution which we find does not reach the theoretically equal level of 80 to 100 postulated by Ege. Some modifying factor must come into play whereby, in effect, equilibrium is established on the basis of a lower sugar concentration in the total water of the corpuscles than in the water of the plasma. The sugar actually within a corpuscle is not necessarily equally distributed through all the water present in such a cell.

The paper by Ege and Roche contains one point of considerable interest to us. These authors show that the fermentable sugar can be easily washed out of the corpuscles while the non-fermentable reducing materials remain fixed in the cells. These findings confirm our experience in the analyses of extracts from unlaked blood.

The unequal distribution of the blood sugar between plasma and corpuscles was found by us just by inspection of the sugar values which we obtained in our comparative analyses. Since the distribution was not included in our original plan of study, the corpuscle values were not determined in these early analyses. The data recorded in Table I represent the analyses of normal blood obtained from 1st year medical students (before breakfast).

TABLE I.

*Showing Normal Fasting Blood Sugar in Laked Blood, Unlaked Blood, and Plasma (by the 1929 Folin-Wu Method). Average Ratio of Corpuscle Sugar to Plasma Sugar Is about 60:100.*

| Subject. | Blood sugar, mg. per cent. | | | $\frac{\text{Corpuscle sugar}}{\text{Plasma sugar}} \times 100.$ |
|---|---|---|---|---|
| | Laked blood. | Unlaked blood. | Plasma. | |
| Adams | 86 | 72 | 86 | 61 |
| Prigot | 84 | 73 | 86 | 64 |
| Murphy | 80 | 68 | 80 | 64 |
| Campbell | 93 | 79 | 94 | 62 |
| Low | 87 | 73 | 87 | 62 |
| Smith | 87 | 74 | 92 | 53 |
| Whelan | 88 | 74 | 93 | 51 |
| Cannon | 92 | 81 | 96 | 63 |
| Miller | 90 | 74 | 92 | 53 |
| Saisen | 100 | 83 | 102 | 58 |
| Hamilton | 93 | 75 | 91 | 58 |
| Dexter | | 81 | 99 | 58 |
| Kelley | | 83 | 100 | 60 |
| A. S. | | 84 | 99 | 64 |
| Forbes | | 89 | 106 | 58 |
| Average | | 78 | 94 | 59 |

From the figures recorded in Table I, it will be seen that the "sugar" in laked normal blood is from 11 to 17 mg. per cent higher than in unlaked blood and that the sugar as found in the unlaked blood is on the average 17 mg. per cent below that of the plasma. The volumes of the corpuscles were not determined for the bloods recorded in Table I, but on the basis of a corpuscle volume of 42 per cent (Rowntree's normal average) we arrive at figures for the corpuscle sugar concentration ranging from 51 per cent to 64 per cent of the simultaneous concentrations found in the plasma, the average ratio being about 60:100.

The glucose distribution in diabetic blood is not quite the same as in the blood of fasting normal persons. The relative concentration in the corpuscles is a little higher, and the average distribution ratio is 69:100 instead of 59:100 as found for the normal blood. The sustained pressure of abnormally high plasma sugar apparently overcomes to a certain extent the resistance of the cells, but out of the twenty-seven diabetic bloods examined we did not find a single one where the concentration of the sugar in the water of the corpuscles reached the level found in plasma. The distribution ratios recorded in Table II are based on corpuscle volumes determined by the hematocrit method. These corpuscle volumes varied between 39.5 and 44 per cent.

TABLE II.

*Illustrating the Distribution of Sugar in Diabetic Blood between Corpuscles and Plasma. Average Ratio Is 69:100.*

| Subject No. | Blood sugar, mg. per cent. | | $\frac{\text{Corpuscle sugar}}{\text{Plasma sugar}} \times 100.$ |
|---|---|---|---|
| | Unlaked blood. | Plasma. | |
| 1 | 230 | 263 | 69 |
| 2 | 213 | 240 | 73 |
| 3 | 124 | 150 | 60 |
| 4 | 370 | 425 | 68 |
| 5 | 159 | 185 | 67 |
| 6 | 124 | 147 | 64 |
| 7 | 270 | 313 | 69 |
| 8 | 159 | 178 | 76 |
| 9 | 171 | 190 | 75 |
| 10 | 164 | 187 | 69 |

Reference has already been made to the fact found by Ege and Roche that the sugar can be easily removed from unlaked blood corpuscles by washing with salt solutions. It seemed to us worth while to make a few analogous experiments just in order to see how nearly the sugar values so obtained from the corpuscles would correspond to the values obtained by calculation from analyses of unlaked whole blood and plasma. In such experiments one would naturally expect the found corpuscle sugar to give somewhat higher values than those obtained by calculation. From the figures of Table III it will be seen that the agreement is surprizingly good.

*2. Urea.*—From the standpoint of distribution between blood cells and plasma, each substance apparently tends to reach its own characteristic ratio. And, of all the common substances, urea is the one which naturally stands out as the most readily distributed not only between plasma and corpuscles but also between blood and tissues, as was shown by Folin and Denis (4), nearly 20 years ago. There is so little reason to question this point that we have

TABLE III.

*Showing That Corpuscle Sugar As Obtained by Complete Direct Extraction Is Nearly Identical with That Obtained by Calculation from the Sugar of Plasma and Unlaked Blood.*

| Subject No. | Corpuscle volume. | Sugar, mg. per cent. | | | |
|---|---|---|---|---|---|
| | | Plasma. | Unlaked blood. | Corpuscles. | |
| | | | | Found. | Calculated. |
| | *per cent* | | | | |
| 1 | 44 | 150 | 124 | 96 | 91 |
| 2 | 43 | 185 | 159 | 135 | 126 |
| 3 | 44 | 147 | 124 | 103 | 96 |
| 4 | 44 | 178 | 160 | 144 | 136 |
| 5 | 41.5 | 192 | 171 | 149 | 142 |
| 6 | 39 | 188 | 164 | 139 | 126 |
| 7 | 50 | 87 | 73 | 64 | 63 |

TABLE IV.

*Showing That Blood Urea Tends to Be Equally Distributed between the Water of Corpuscles and Plasma.*

| Subject No. | Urea N, mg. per cent. | | $\frac{\text{Corpuscle urea}}{\text{Plasma urea}} \times 100$. |
|---|---|---|---|
| | Unlaked blood. | Plasma. | |
| 1 | 12.9 | 14.0 | 82 |
| 2 | 12.3 | 12.9 | 88 |
| 3 | 16 7 | 18 7 | 75 |
| 4 | 11.7 | 12.3 | 89 |
| 5 | 9.4 | 10.5 | 75 |
| 6 | 13.3 | 14.5 | 81 |
| 7 | 14.7 | 16.0 | 80 |
| 8 | 12.5 | 14.0 | 75 |
| 9 | 13.3 | 14.8 | 76 |
| 10 | 13.3 | 14.9 | 75 |

not considered it worth while to make any special experiments to prove it. In Table IV we give the data obtained in the course of the comprehensive analyses involving no repetitions, yet we see here that the average ratio must come very near to the proportion of 80:100 which, as pointed out by Ege, would signify equal concentration in the water of the plasma and of the cells. In each case the corpuscle volume is assumed to be 42 per cent.

*3. Creatinine.*—For creatinine as well as for urea Folin and Denis found a rapid and equal distribution between blood and tissues in the course of injection experiments with creatinine, and it therefore seems probable that the distribution between corpuscles and plasma should be very near to the point of equality represented by the ratio 80:100. But one can scarcely hope to secure analytical values for the small amounts of preformed creatinine in blood which shall be so accurate as to exclude rather large variations. From the figures given in Table V it will be seen, however, that the ratios for the creatinine distribution come as close as could be expected to the ratio of equal distribution (80:100).

TABLE V.

*Showing That Blood Creatinine Probably Tends to Be Equally Distributed between the Water of Corpuscles and Plasma.*

| Subject No. | Creatinine, mg. per cent. | | $\frac{\text{Corpuscle creatinine}}{\text{Plasma creatinine}} \times 100.$ |
|---|---|---|---|
| | Unlaked blood. | Plasma. | |
| 1 | 1.07 | 1.25 | 65 |
| 2 | 1.17 | 1.18 | 98 |
| 3 | 1.20 | 1.25 | 90 |
| 4 | 1.08 | 1.18 | 80 |
| 5 | 1.02 | 1.20 | 64 |
| 6 | 1.09 | 1.20 | 77 |
| 7 | 1.05 | 1.25 | 62 |
| 8 | 1.07 | 1.20 | 75 |
| 9 | 1.03 | 1.25 | 58 |
| 10 | 1.11 | 1.25 | 74 |

Earlier studies of the creatinine distribution have given extremely erroneous results. Wu (5), for example, found nearly 6 times as much "total creatinine" in the corpuscles as in the plasma. These relatively huge errors are due to the fact that disintegrated blood corpuscles contain substances which react with the alkaline picrate reagent, as they do with sugar reagents, and the effect is relatively even more disastrous, because the amount of true creatinine present is so small. The creatinine values obtained on whole blood, therefore, always have been larger than the values obtained on plasma, whereas they should be smaller. Here we have, therefore, another striking illustration of the fact that the universally used filtrates obtained from laked blood have always given more or less misleading results. (Another equally striking illustration will be found in the case of the amino acids.)

The creatinine figures in Table V show incidentally that the creatinine content of normal (student) blood is just a little over 1 mg. per cent.

*4. Uric Acid.*—Folin, Berglund, and Derick (6) found, in the course of a great many analyses, that the distribution to tissues of injected uric acid must be very small indeed. And we now find a more or less similar situation with reference to the distribution of uric acid normally present in blood between corpuscles and plasma. Reasonably dependable studies of the distribution of uric acid have now become possible, whereas all earlier studies could give only erroneous results. In Wu's analyses the uric acid concentration in the corpuscles varies between 40 and 67 per cent of that found in plasma. According to our analyses, the true normal ratio seems to be about 22:100.

Moreover, our analyses indicate that the distribution ratio for uric acid is remarkably constant and that when marked deviations are found these are probably due to analytical errors. Comparatively small errors in the analytical figures may lead to relatively huge changes in the distribution ratio. For example, if the true uric acid in unlaked blood and plasma is respectively 2.7 and 4.0 mg. per cent and the corpuscle volume 42 per cent, we obtain a distribution ratio of 23:100. But if the found uric acid values are 2.9 and 3.8 mg. per cent the distribution ratio would jump to 45:100, and if the two analytical errors (of 0.2 mg. per cent) were in opposite direction, the distribution ratio would become 3.6:100.

The first six bloods recorded in Table VI had been subjected to complete analyses without any duplicate uric acid determinations, and before we had given a thought to the question of how the uric acid is distributed between corpuscles and plasma. Four other blood analyses, made under the same conditions, gave us, however, one low distribution ratio of 12:100 and one high one of 38:100. If these four analyses were included, we would get an average distribution ratio of 25:100 for uric acid. But such an average figure has little or no value if it must include ratios of 12:100 and 38:100. Being convinced that these extreme ratios were due to avoidable analytical inaccuracies we have replaced these analyses by new ones on bloods from the same persons and have thus obtained the last four sets of figures recorded in Table VI.

There can be little doubt about the fact that the distribution of uric acid between corpuscles and plasma is normally subject to only slight variations.

TABLE VI.

*Illustrating the Uric Acid Content of Normal Fasting Student Blood and the Distribution of Uric Acid between Corpuscles and Plasma.*

| Subject. | Blood uric acid, mg. per cent. | | $\frac{\text{Corpuscle uric acid}}{\text{Plasma uric acid}} \times 100.$ |
|---|---|---|---|
| | Unlaked blood. | Plasma. | |
| Simmons | 2.9 | 4.3 | 22 |
| Smith | 2 7 | 4.0 | 23 |
| Whelan | 2.8 | 4.1 | 24 |
| Cannon | 2.7 | 4.0 | 23 |
| Prigot | 3.2 | 4 8 | 21 |
| Murphy | 2.3 | 3.4 | 23 |
| Adams | 2.2 | 3.3 | 21 |
| Campbell | 2.7 | 4.0 | 22 |
| Low | 2.3 | 3.5 | 22 |
| Hamilton | 2.4 | 3.5 | 23 |

Incidentally, it may be pointed out that the study of distribution ratios for substances present in small amounts in the blood constitutes an extremely severe test of the dependability of the analytical method employed.

*5. Amino Acids.*—All earlier analyses have yielded higher amino acid values for whole blood than for plasma, and according to these results the concentration in the corpuscles should be very much greater than in the plasma. Folin and Denis, as well as Van Slyke likewise found higher concentrations in tissues than in blood after the administration of amino acids. A very high distribution ratio for this nitrogen seemed, therefore, not unreasonable. The analytical determination of this nitrogen is necessarily a little less definite than others because it represents a whole large group of different individual compounds; for this same reason the distribution ratio might also be subject to unusually large variations. The significance of the analytical figures recorded in Table VII is, however, unmistakable. The diffusible amino acid nitrogen is much more abundant in the plasma than in the intact corpuscles and previously published results have been quite misleading. The preliminary laking of blood evidently exposes an abundance of reacting groups which may have no free existence in the living cells and which probably have nothing to do with blood as a transportation system.

TABLE VII.

*Showing That Diffusible Amino Acid Nitrogen Is Much More Abundant in Plasma Than in Corpuscles.*

| Subject. | Amino acid N, mg. per cent. | | Amino acid N. |
|---|---|---|---|
| | Unlaked blood. | Plasma. | $\frac{\text{Corpuscles}}{\text{Plasma}} \times 100.$ |
| Simmons | 5.6 | 7.1 | 50 |
| Smith | 5.0 | 7.8 | 14 |
| Whelan | 5 1 | 7.6 | 22 |
| Prigot | 5.3 | 7.4 | 32 |
| Murphy | 5.0 | 7.5 | 21 |
| Campbell | 5.4 | 8.0 | 22 |
| Low | 5.6 | 7.1 | 50 |
| Hamilton | 5.9 | 8.2 | 33 |

*Amount and Composition of the Non-Protein Nitrogen in Unlaked Blood.*—The analytical figures published in the first paper on the preparation of unlaked blood extracts show that the total non-protein nitrogen is normally about 10 mg. per cent lower than the nitrogen obtained from laked blood filtrates. It seemed practically certain that nearly the whole of this large difference would be found to be represented by the obscure undetermined nitrogen or rest nitrogen, but it was necessary to show by comprehensive analyses to what extent this is actually the case. From the analytical figures given in this section it will be seen that the undetermined nitrogen has disappeared when we work with unlaked blood extracts. The sum of the nitrogen from the urea, uric acid, creatinine, and amino acids adds up to practically 100 per cent of the total nitrogen.

With respect to the absence of undetermined nitrogen our unlaked blood extracts are just like plasma extracts which, according to our analyses, as well as the earlier analyses of Wu, are also substantially free from unknown nitrogenous materials.

The absence of the indeterminate nitrogen derived from disintegrated blood cells leaves so little rest nitrogen in the case of normal human blood that it falls within the limits of unavoidable analytical errors. But this condition does not necessarily obtain in bloods of other animals or in abnormal human blood. In nephritic bloods with accumulating nitrogen retentions there must inevitably also accumulate appreciable quantities of nitrogenous products which would be covered by the term undetermined nitrogen. And it is quite possible that in other pathological conditions one might also be able to find some undetermined nitrogen.

Comprehensive analyses of unlaked blood, plasma, and laked blood were made on nineteen students, in ten of whom the bloods were taken before breakfast and in nine about 2½ hours after breakfast. The tables representing all of these analyses will be kept on file for some possible future use, but at present it would seem sufficient to give the data in the form of one condensed table.

From the figures given in Table VIII it would appear that for nitrogen determinations it makes practically no difference whether the blood is taken before, or 2½ hours after, breakfast. The amino acid nitrogen and the uric acid tend to be somewhat higher in the bloods taken after breakfast while for the urea the tendency seems to be in the opposite direction. But the differences are so small that for clinical purposes, at least, it should make no perceptible difference whether the blood is taken before or after breakfast.

The figures of Table VIII serve to illustrate the limits of variation for the different nitrogenous products in unlaked blood filtrates from the blood of strictly normal young adults. It may be noted that for unlaked blood the non-protein nitrogen varies between 13.8 and 20.8 mg. per cent, whereas for the laked blood the variation lies between 25.6 and 42.0 mg. per cent. For unlaked blood, the urea nitrogen represents about 68 per cent of the total nitrogen, whereas for laked blood it represents only about 40 per cent.

Since the non-protein nitrogen of laked blood is not only much larger, but also more variable than the nitrogen from unlaked blood, it seems fairly certain that the latter should supply a better basis for the diagnosis of beginning abnormal nitrogen retention.

TABLE VIII.

*Giving a Highly Condensed Summary of the Composition of 57 Blood Filtrates Obtained from Nineteen Normal Persons (Students).*

All figures are expressed as mg. per cent.

| | | Urea N. | Amino acid N. | Uric acid. | Creatinine. | Total N. | Rest N. |
|---|---|---|---|---|---|---|---|
| Before breakfast. | | | | | | | |
| Unlaked blood. | Highest. | 14.2 | 5.6 | 3.2 | 1.20 | 20.8 | |
| | Lowest. | 9.4 | 4.2 | 2.2 | 1.03 | 13.8 | |
| | Average. | 12.6 | 5.0 | 2.6 | 1.09 | 18.5 | (−) 0.4 |
| Plasma. | Highest. | 16.0 | 7.9 | 4.8 | 1.25 | 25.0 | |
| | Lowest. | 10.5 | 6.7 | 3.3 | 1.18 | 19.1 | |
| | Average. | 13.8 | 7.5 | 3.9 | 1.22 | 22.6 | (−) 0.5 |
| Laked blood. | Highest. | 14.5 | 10.2 | 3.3 | 1.45 | 35.3 | |
| | Lowest. | 9.8 | 7.8 | 2.2 | 1.20 | 25.6 | |
| | Average. | 13.0 | 8.7 | 2.5 | 1.38 | 30.7 | 7.6 |
| 2½ hrs. after breakfast. | | | | | | | |
| Unlaked blood. | Highest. | 13.3 | 5.9 | 3.5 | 1.05 | 20.2 | |
| | Lowest. | 7.7 | 3.9 | 2.5 | 0.91 | 14.6 | |
| | Average. | 11.5 | 5.1 | 3.0 | 0.98 | 18.0 | 0 |
| Plasma. | Highest. | 16.5 | 9.7 | 5.5 | 1.33 | 28.3 | |
| | Lowest. | 10.7 | 7.0 | 3.7 | 1.07 | 20.2 | |
| | Average. | 13.3 | 8.7 | 4.5 | 1.18 | 24.2 | 0.2 |
| Laked blood. | Highest. | 14.2 | 10.4 | 3.7 | 1.41 | 42.0 | |
| | Lowest. | 8.0 | 8.3 | 2.4 | 1.18 | 26.4 | |
| | Average. | 11.8 | 9.5 | 3.0 | 1.28 | 30.8 | 8.0 |

This is an important and alluring, but also an elusive topic. It is doubtless quite true that normal kidneys possess much surplus capacity for the excretion of waste products, but this does not necessarily mean that the kidneys are also very responsive to moderate increases in the concentration of such a circulating waste product as urea. We have satisfied ourselves by special experiments that such is not the case. We have raised the level of the urea nitrogen 5 to 10 mg. per cent by the administration of small amounts of urea, and under these conditions have found that these higher levels remain practically unchanged for several hours. It therefore seems probable that up to 31 or 32 mg. per cent of non-protein nitrogen in unlaked blood might occur without signifying abnormal nitrogen retention. On the other hand, in normal persons such high levels of non-protein nitrogen with not less than 22 mg. per cent of urea nitrogen, should occur only on the basis of unusually heavy protein consumption.

TABLE IX.

*Giving a Comparison of Unlaked and Laked Blood in Cases of Mild or Moderate Retention.*

| Subject No. | Unlaked blood. |  | Laked blood. |  |
|---|---|---|---|---|
|  | Non-protein N. | Urea N. | Non-protein N. | Urea N. |
|  | *mg. per cent* | *mg. per cent* | *mg. per cent* | *mg. per cent* |
| 1 | 30.0 | 20.0 | 41.0 | 20.4 |
| 2 | 29.6 | 21.0 | 38.7 | 21.6 |
| 3 | 36.0 | 23.0 | 44.0 | 25.0 |
| 4 | 36.4 | 25.0 | 44.0 | 25.3 |
| 5 | 41.0 | 31.8 | 52.0 | 30.8 |
| 6 | 69.0 | 53.0 | 77.0 | 56.0 |
| 7 | 71.0 | 56.0 | 80.0 | 62.0 |

In many, perhaps in most cases, the finding of even 25 mg. per cent of non-protein nitrogen might readily be due to beginning abnormal retention.

In Table IX are given a few analyses of bloods obtained from patients at the Peter Bent Brigham Hospital. Though few in number, these analyses become rather instructive when compared with the normal values recorded in Table VIII. In cases of quite unmistakable nitrogen retentions, as shown by Subjects 5, 6, and 7, it obviously makes practically no difference whether laked or unlaked blood is used for the analyses. When the non-protein nitrogen has risen to 40 or over in unlaked blood or to 50 and upwards in laked blood the existence of nitrogen retention is, of course, established. But in the first four subjects, the non-protein nitrogen in the laked blood has not risen above 44 mg. per cent, and this is only 2 mg. per cent above the highest normal figure recorded in Table VIII. In all of these four cases one, therefore, would be rather uncertain, although the accompanying high urea nitrogen values certainly indicate abnormal nitrogen retention. The same bloods when analyzed without previous laking, give clearly a much more positive indication of nitrogen retention when comparison is made on the basis of the non-protein nitrogen. As against the highest normal value in Table VIII of 20.8 mg. per cent, the four hospital bloods have yielded 30, 29.6, 36, and 36.4 mg. per cent.

These figures are cited merely to show that unlaked blood cannot fail to be more useful than laked blood in clinical applications of blood analysis.

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# A VACUUM TUBE POTENTIOMETER APPLICABLE FOR USE WITH GLASS ELECTRODES OF HIGH RESISTANCE.

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A paper was published last August by Stadie[1] describing an electron tube potentiometer, and Stadie's potentiometer was tried out in Cambridge. It did not prove very satisfactory owing to difficulty of adjustment and to drift. The potentiometer described in this paper has been developed by a step by step evolution from Stadie's. The result is a less complicated potentiometer and the elimination of drift. The present potentiometer can be used with sufficient accuracy when the resistance of the glass electrode is as high as 100,000 megohms.

This development depended upon two very useful characteristics of the screen grid tube. The first is that if the screen grid voltage is sufficiently low, about 4 volts, the plate current can be carried directly through a sensitive galvanometer with good control of galvanometer deflection by adjustment of the control grid voltage (see Fig. 1, curves *A*, *B*, *C*, and *D*). This eliminates the complication of balancing off the plate current when one is using a galvanometer.

The other characteristic is that, under the same conditions as above, the control grid can be entirely disconnected and the galvanometer deflection can then be controlled by adjustment of the screen grid voltage (see Fig. 1, curve *E*).

If these two characteristics are considered in combination, it is evident that we may perform the following operation. With the control grid open we can adjust the screen grid voltage and bring

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[1] Stadie, W. C., *J. Biol. Chem.*, **83**, 477 (1929).

the galvanometer deflection to any convenient value. Call this convenient deflection Δ. Now without changing the adjustment of the screen grid we can close the control grid circuit and, by adjustment of the control grid voltage, we can again bring the

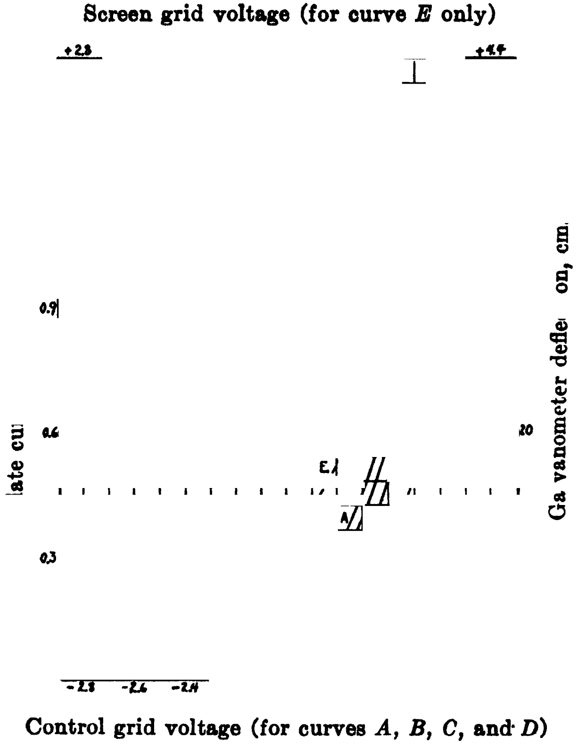

FIG. 1. Characteristic curves of screen grid tube *UX222*. Plate voltage, 90; filament voltage 2. Curve *A* taken with screen grid at + 4.16 volts, curve *B* at + 4 volts, curve *C* at + 3.8 volts, curve *D* at + 3.4 volts; curve *E* taken with control grid open-circuited.

galvanometer to deflection Δ. When this has been done the control grid may be opened and closed without change of galvanometer deflection, and the tube may be considered as balanced with respect to the control grid voltage.

To appreciate fully the significance of this, we should understand the rather complex action going on inside of a working tube.

Without going into too much detail it will be sufficient to state that due both to the electron stream flow and to the surface leakages, internal and external, a disconnected grid in a tube tends to assume some potential depending upon its position in the tube. If a connection is made from that grid to an external voltage so that the potential of the grid is in any way changed, current will flow through that connection and will continue to flow as long as the new grid potential is maintained. Conversely, if a connection to an external voltage can be made without change of grid potential no current will flow through the connection. A change of grid potential is indicated by a change of plate current and it follows that if we can adjust the two grid potentials of a screen grid tube so that the plate current does not change when the control grid circuit is opened and closed, no current will flow in the closed control grid circuit. It also follows that for every other adjustment of the control grid voltage, current will flow in the control grid circuit.

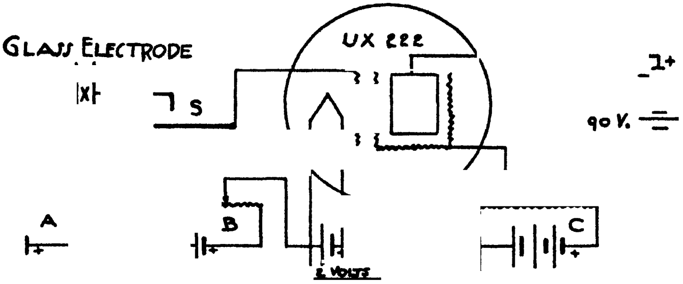

FIG. 2. Simplified wiring diagram.

The drift found when Stadie's potentiometer was used was undoubtedly due to flow of current in the grid circuit, as Stadie could not comply with the conditions of balance as stated above. As the glass electrode was in the control grid circuit, and the current flow passed through the glass, it naturally affected the voltage of the glass electrode.

Stadie realized that at a certain grid voltage the condition of balance occurred, but he did not have the point of balance under control and he found that with high resistance in the grid circuit his potentiometer was insensitive when at the point of balance. That the potentiometer described in this paper is not insensitive at the point of balance, when used with high resistance in the control grid circuit, may be linked with the fact that the electron flow in the tube is about $\frac{1}{1000}$ that used in Stadie's potentiometer.

Reducing the plate current from milliamperes to microamperes not only reduces the grid current for unbalanced grid, but it also improves the stability of the tube and eliminates much of the delay of waiting for the tube to become stable.

The simplest circuit utilizing this principle of tube balance is given in Fig. 2. Note that this arrangement involves three ordinary resistance type potentiometers, *A*, *B*, and *C*, but of these only *A* is calibrated; *B* and *C* are quite simple. To use the apparatus, switch *S* is first set in the mid-position, so that the control grid circuit is open, and the deflection of the galvanometer is adjusted by potentiometer *C*, to some convenient deflection, Δ. Switch *S* is next thrown down and potentiometer *B* is adjusted to return the deflection to Δ. Then switch *S* is thrown up and potentiometer *A* is adjusted to return the deflection to Δ. The voltage of potentiometer *A* is then equal and opposite in direction to the voltage of the unknown.

In this circuit the tube, the glass electrode *X*, and switch *S* must be enclosed in a metal-lined and grounded box.

Actual wiring diagrams of the apparatus are given in Figs. 3 and 4. *A*, *B*, and *C* are again the three resistance potentiometers, now shown in detail. In Fig. 3 potentiometer *A* is a Leeds and Northrup student potentiometer (No. 7651) and in Fig. 4 is a Leeds and Northrup type K potentiometer.

Switch *E* shifts the galvanometer from the plate circuit of the tube to potentiometer *A* for adjusting *A*. Switch *D* closes before switch *E* opens, so that the plate current is not interrupted. Switches *D* and *E* are mechanically interconnected.

Potentiometer *B* was made from three General Radio Company standard radio potentiometers connected as shown. The 1800 ohm unit (type 371) gives rough adjustment, the 400 ohm unit (type 214) rather fine adjustment, and the 5 ohm unit (type 371) gives very fine adjustment. An extra contact arm was fitted on the 400 ohm unit to give the two contacts as shown close to each

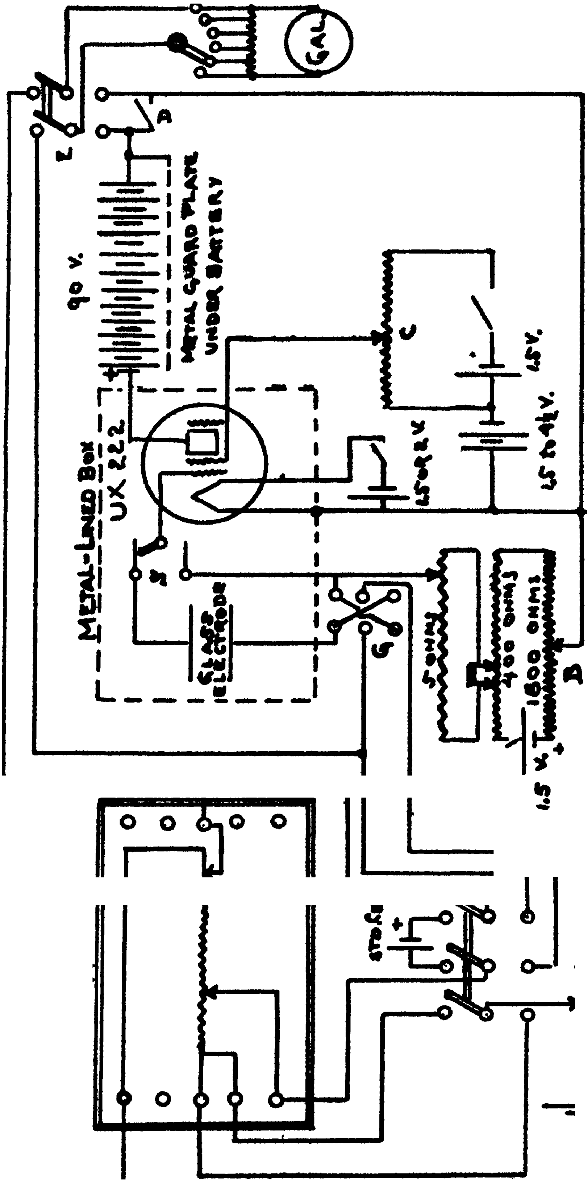

FIG. 3. ng diagram with a Leeds and Northrup student potentiometer, No. 7651.

other; these are insulated from each other but are moved together by a single knob. The 5 ohm unit is connected between these two contact arms.

Potentiometer *C* is a single 5000 ohm (type 371) General Radio Company potentiometer.

Switch *G* is a reversing switch for potentiometer *A*.

The 90 volt battery is radio type and should be well insulated from ground.

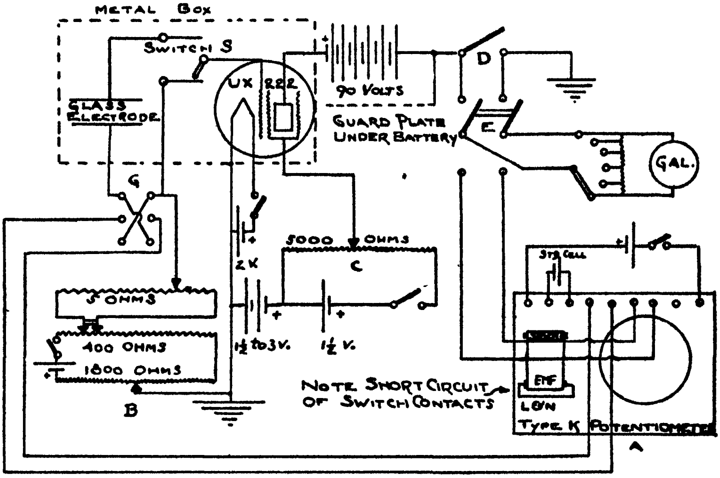

FIG. 4. Wiring diagram with a Leeds and Northrup type K potentiometer.

Much depends upon the design of switch *S*. The form shown in Fig. 5 has proved satisfactory.

The galvanometer used was Leeds and Northrup type R (No. 2500e), sensitivity 0.003 microamperes per mm. The metal lining of the box should be well soldered and grounded. It does not seem possible to obtain satisfactory shielding with a window in the box even though the window is covered with copper netting soldered on all edges and well grounded.

Dry cells may be used, but for potentiometer *A* and the tube it is best to use large, new storage cells.

Fig. 6 is a photograph of the metal-lined box. The knob of switch *S* is to the right of the door. Potentiometers *B* and *C* and

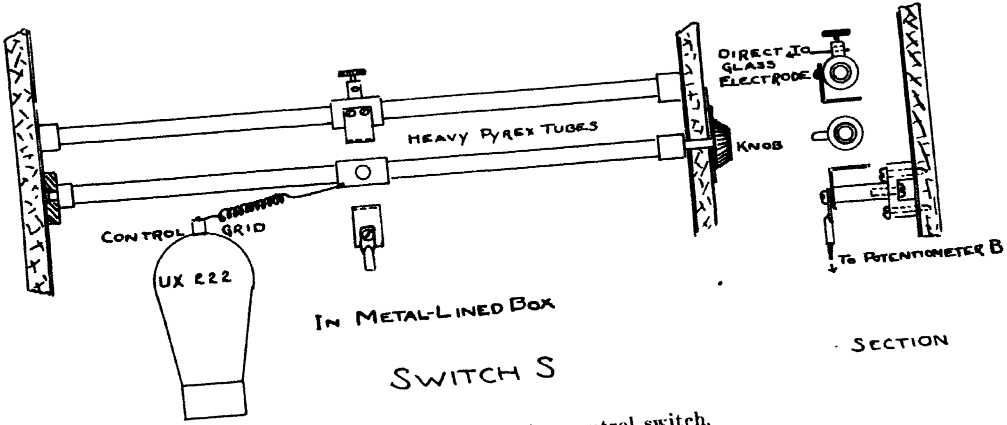

FIG. 5. High insulation control switch.

switches $D$, $E$, and $G$ are in a sub-base compartment. The binding posts are on a panel of this compartment at the back of the box and the battery snap switches are on the box top. On one side of the box is an electric hair dryer mounted to draw air from the top of the box and deliver it into the sub-base. From the sub-base the air passes up through holes into the metal-lined compartment and these holes are inclined at an angle to give the air in the box a rotary motion. The heater in the hair dryer is connected through

Fig. 6. Photograph of cabinet.

a mercury vacuum tube switch which is controlled by a thermostat. Thus the temperature of the box is under control.

On either side of the box just inside of the door are two vertical Pyrex tubes which carry the ring brackets for the calomel half-cells. These brackets can be easily slid up and down and turned, but stay in any desired position. Their construction is shown in Fig. 7. The tubes also can be moved up and down by knurled nuts on top of the box so that the final connection with the glass electrode can be made with micrometer accuracy. The long Pyrex tubes give the high insulation that is necessary.

The calomel half-cells, one of which is shown in Fig. 7, can be turned and tilted in the rubber-covered rings to bring the tips to any desired position. The stop-cocks are greased only at the edges. The glass cap tips are as described by Kerridge.[2] These caps should be removed, washed in KCl from the bulb, replaced, washed in distilled water, and dried with filter paper before each pH determination.

The sensitivity of the potentiometer under different conditions of resistance can be calculated from the curves of Fig. 8. Sensitivity may be expressed as the slope of these curves at the point where the curves cross and this may be expressed as the change of the potentiometer voltage for a given change of galvanometer deflection. The galvanometer can be balanced within approximately 0.2 mm. deflection (0.0006 microamperes in the galvanometer used) and for this error in deflection from the correct balance the errors of voltage reading are as follows:

| Resistance in control grid circuit. | Change in potentiometer voltage for 0.2 mm. deflection of galvanometer from correct balance. | Corresponding pH error. | Sensitivity. |
|---|---|---|---|
| *megohms* | *volt* | | *mm. per volt* |
| 0 | 0.00018 | 0.003 | 1100 |
| 10,000 | 0.00023 | 0.004 | 870 |
| 100,000 | 0.00058 | 0.01 | 345 |

These high resistances were made of soft glass tubes containing mercury and standing in mercury. The 100,000 megohm unit

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[2] Kerridge, *J. Sc. Instruments*, 3, No. 12 (1926).

may be as much as 50 per cent off in resistance, as the total galvanometer deflection when measuring its resistance was only an estimated 0.3 mm. The 10,000 megohm resistance is more nearly correct but still only approximate. It was adjusted to a galvanometer deflection of 3 mm.

The value of the control grid current for any unbalanced adjustment can also be estimated by means of these curves. For any galvanometer deflection let $V$ be the control grid voltage as read from curve $A$ and let $v$ be the control grid voltage as read from curve $B$. Then the control grid current will be $\dfrac{V - v}{10{,}000{,}000{,}000}$ amperes. The sensitivity figures indicate that if the control grid adjustment is within 0.2 mm. of the balance, the grid current will be approximately $5 \times 10^{-15}$ amperes.

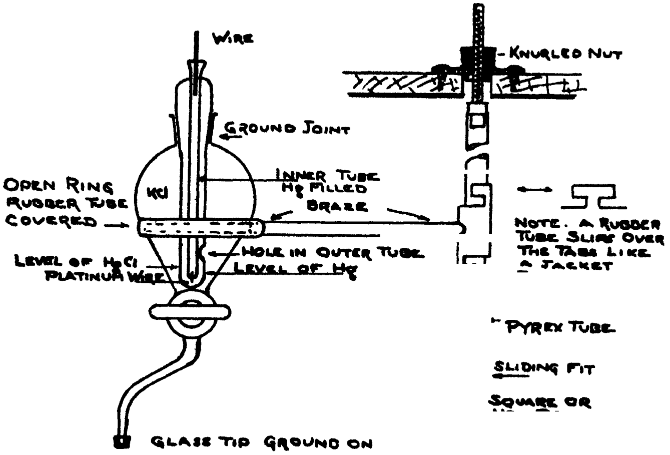

FIG. 7. Calomel half-cell and bracket.

As the control grid voltage is made more negative, the grid current increases to a maximum value of about $3 \times 10^{-9}$ amperes and then decreases. This decrease is due to the falling off of the electron flow in the tube. The indications from shape of the curves are that a still more sensitive galvanometer would not increase the sensitivity but would further reduce grid current when unbalanced.

Checks of sensitivity were made, with a type K potentiometer for potentiometer *A*, by measuring the voltage of a standard cell through the 100,000 megohm resistance. The voltage of the standard cell was actually 1.01846. This was measured with the resistance in series once as 1.01866 volts and again as 1.01805.

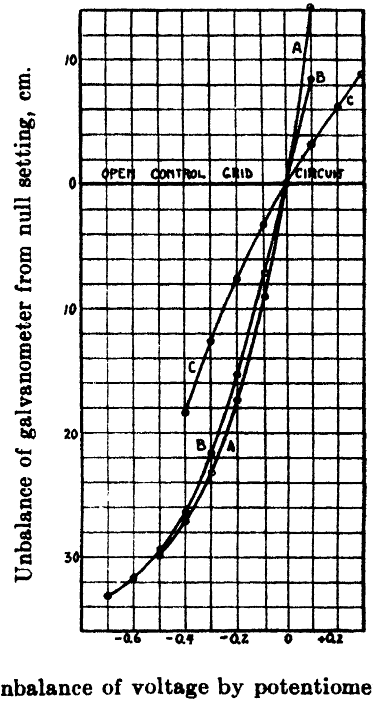

Fig. 8. Sensitivity curves. Curve *A*, no resistance in control grid circuit; curve *B*, 10,000 megohms resistance; curve *C*, 100,000 megohms resistance.

These readings were complicated by the fact that the glass itself had a slowly changing voltage.

Typical readings of a 500 megohm glass electrode voltage are as follows: 0.14979, 0.14989, 0.14989, 0.14975, 0.14986, 0.14986 volt.

As stated above the tube is remarkably stable, due to the small plate current. To illustrate this a test was made, with a glass electrode, to see how long it took before reliable readings could be obtained after switching on the tube and potentiometers. The first reading was made 2½ minutes after the start and the subsequent readings at about the same time interval; the results are as follows: 0.17551, 0.17602, 0.17601, 0.17607 volt. The reading after 2½ minutes was 0.0005 volt low, corresponding to an error of 0.008 pH.

Following this test the time taken to change the tube and reach stability with a new tube was tested. The first reading, taken 2½ minutes from the time of starting to change tubes, was 0.17554 volt. 2½ minutes later a reading of 0.17604 volt was obtained, indicating that stability had been already reached.

Glass electrodes usually (or always) show some voltage or charge in addition to that accounted for by the pH differences of the liquids of the cell. It is, therefore, impossible to determine pH difference directly by one reading of voltage. Consequently, we must use a method of substitution. Thus if $A$, $B$, and $C$ are three liquids and if the pH of $B$ is known ($\mathrm{pH}_B$), we may make a voltage reading with $A$ and $B$ in the glass electrode cell and then with $A$ and $C$. Call these two readings $V_{ab}$ and $V_{ac}$.

The pH of liquid $C$ will be

$$\mathrm{pH}_c = \mathrm{pH}_B \pm \frac{V_{ab} \pm V_{ac}}{0.059} \text{ for } 25°.$$

Note that English screen grid tubes are not very suitable for this potentiometer because in England the control grid is brought to a prong instead of to the top of the tube where the insulation resistance is highest. Undoubtedly this potentiometer is suitable for other uses besides that of pH measurement. It should prove useful in measuring voltage whenever an ordinary potentiometer is insensitive due to high resistance in the circuit to be measured or when it is prohibitive to draw appreciable current when balancing.

Although the statement is made above that the potentiometer is capable of measuring a glass electrode voltage when the resistance is as high as 100,000 megohms, this does not imply that it is advisable to use such electrodes.

## SUMMARY.

A potentiometer is described in which a single screen grid vacuum tube is used. Plate current is so reduced, by lowering the screen grid voltage, that it can be carried directly through a sensitive galvanometer. The tube is first balanced by proper adjustments of the grid voltages until a working condition of zero control grid current is obtained, and then the cell of unknown voltage and a standard potentiometer are included in the control grid circuit. When the balance of the tube is reestablished, the voltage of the unknown may be read directly by the standard potentiometer.

This potentiometer is sufficiently sensitive when used with resistances as high as 100,000 megohms for accurate pH determination. The potentiometer when adjusted to a reasonable accuracy takes less than $5 \times 10^{-15}$ amperes from the glass electrode and this low current causes no drift of glass electrode voltage. Stable conditions of the tube occur in less than 5 minutes from switching on the batteries.

# THE DETERMINATION OF PEPTIC ACTIVITY: AN EXAMINATION AND APPLICATION OF THE GATES METHOD OF PROTEOLYTIC ENZYME TITRATION.

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Despite the numerous methods for the determination of proteolytic enzymes, there are few, if any, which incorporate all the advantages of accuracy, simplicity, and brevity, and which require only a small sample for a determination. In 1927 Gates (1) published a preliminary report of a proteolytic enzyme titration possessing these desirable features, and demonstrated his method at the 1928 meeting of the American Societies for Experimental Biology held at Ann Arbor. This method has been extensively investigated in this laboratory and found to be most valuable for rapid and accurate determination of peptic activity. The principles of the method as outlined by Gates have been retained. However, the authors have to some extent modified the manipulative details, and have supplemented the outline of the procedure as presented in the preliminary report to include an accurate means of quantitative estimation of peptic activity.

## *Principle of Method.*

The Gates method involves the digestion of the gelatin layer of a photographic film. The surface of a film is composed of a uniform layer of gelatin in which silver is evenly dispersed. Upon reduction of the film by exposure and subsequent development of the film, an almost opaque substrate results. Digestion of the gelatin liberates reduced silver with a progressive change from opacity to transparency, depending in degree upon the extent of the digestion. Proteolytic activity is determined by an estimation of the difference in the intensity of the light penetrating the film before and after digestion.

## *Procedure.*

*Preparation of Films.*—The choice of films is optional. Of the two types tested, Eastman's commercial and commercial-ortho, the latter was found to be the more sensitive for the estimation of peptic activity. A film 8 inches by 10 inches is a convenient size to use. To insure complete and uniform reduction, the films are exposed to Roentgen rays, 25,000 volts, 10 milliamperes, at a target range of 24 inches for a period of 2 minutes. A pile of six films can be exposed at the same time. The films are then developed and fixed in plain sodium hyposulfite, no hardener being used. As the opacity of the film will depend upon the time that the film is allowed to remain in the developer, it is well to standardize this procedure. The opacity of the film depends entirely upon the extent of the reduction of the silver. The amount of gelatin on the films is not influenced by exposure or development. Therefore a dark film will favor more accurate results, inasmuch as the greater the opacity, the greater will be the change in penetrability of light through the film per unit of gelatin digested. On the other hand, if the films are allowed to become too dark it will be impossible to measure the slight amount of light penetrating. Films developed until the red light in the developing room is not discernible through them give optimal results. Rewashing of the developed films with distilled water and again drying before use is advantageous in order to "set" the film so that subsequent wettings are without effect After development the large films are cut into smaller units, ¾ inch by 1 inch, to be used for digestive tests.

*Digestion of Films.*—The films are digested in cells, made by mounting rings of No. 14 copper wire, ½ inch in diameter, upon glass squares 1 inch by 1 inch by means of paraffin. This forms a small chamber, the capacity of which is approximately 0.5 cc. The cell is so filled with the solution under examination that the meniscus of the fluid projects above the copper ring. The film to be digested is now placed on top of this cell, the gelatin layer downward and in intimate contact with the enzyme solution. Inasmuch as the film forms the roof of the digestion chamber, the silver, when liberated, will fall to the bottom, thus not interfering with the course of the reaction. Another glass square is placed over this film, and the digestion cell, now complete, is firmly held together with an ordinary spring clothes-pin, and is immersed in a constant temperature water bath for a carefully timed interval. This digestion cell, when carefully constructed, will be absolutely water-tight, allowing no dilution of the enzyme solution when placed in a water bath. A carefully controlled temperature is most important because the temperature coefficient of the reaction is extremely high. A water bath, constant to 0.02° has been found to be the most satisfactory means of temperature regulation. At the conclusion of the digestion the films are carefully washed free of enzyme by immersion in the water bath and dried in a current of air.

*Temperature and pH Regulation.*—If the digestion were allowed to proceed at optimal temperature, the minute amount of gelatin present on the film would be completely removed in a few minutes. To prolong the time of digestion and reduce the error in timing, the films are digested at 25°. At this temperature the gelatin remains hard and firm and at the completion of the reaction presents a smooth, even appearance. Even at this low temperature the films would be completely digested in the 10 minutes by the concentration of pepsin normally found in gastric juice. This offers an opportunity for the dilution of the specimen to be determined with a buffer solution, thus bringing all the samples analyzed to the same pH and reducing still more the amount necessary for a single determination. In a series of peptic digestions carried out at acidities ranging from pH 1 to 4, a pH of 2 was found to be most favorable. Sørensen's glycine-NaCl-HCl buffer solution (2) is the choice at this high acidity.

*Measurement of Light Penetrability.*—To measure the light penetrating before and after digestion, a Klett or other Duboscq type of colorimeter is used. The films are read against a gelatin-silver suspension prepared by dissolving the gelatin layer from two films in about 2 cc. of hot water, filtering through filter paper to remove any large heavy particles, and suspending this black silver emulsion in sufficient glycerol to fill the cup of the colorimeter. The viscous properties of the glycerol will prevent any settling of the silver within the course of several hours. Below the cup of the colorimeter containing this gelatin-silver suspension is placed one of the films from which the gelatin and silver have been removed. The other cup contains glycerol to which has been added the same proportional amount of water as that present in the gelatin-silver suspension just described; below it is placed the film that is being measured. Very accurate determinations of the light coming through the unknown film can be made by regulating the depth of the suspension on the opposite side of the colorimeter until the light intensities match. The films are carried between two thin layers of sheet brass, lacquered flat black in order to avoid internal reflection, and having a hole of ½ inch diameter centrally bored to allow exposure of the digested area of the film. The film carriers are clamped to the rack of the colorimeter by means of a simple paper clip in such a way as to allow the exposed portion of the film to be directly under the cup.

The films are read before and after digestion and the difference in the reading is a measurement of the proteolytic activity of the solution used in their digestion. It will be found that despite careful exposure and development the films will vary in their initial readings. To exclude any variable, it is best when making a series of determinations, to pick a group of films having the same initial reading.

*Sensitivity.*—An indication of the sensitivity of this method can best be demonstrated by a typical digestion curve showing the variation in films acted upon by different concentrations of a commercial enzyme solution. Fig. 1 shows the course of a representative digestion, the concentration of enzyme being variable and the time constant.

As can be seen from Fig. 1, this method is sensitive to but slight changes in concentration and can be used for the quantitative estimation of extremely dilute solutions of pepsin. The curve in Fig. 1, because of its logarithmic character, suggests that the underlying reaction is monomolecular in character. A plot of the reading difference against the logarithm of the enzyme concentration (Fig. 2) supports this view.

In view of the facts that (*a*) the temperature of the reaction is low, and the time of reaction short, thus minimizing enzymatic decomposition, (*b*) the amount of substrate is so small that except in very extreme dilutions there is a relatively large predominance of enzyme and therefore less inhibition due to the end-products of the reaction, and (*c*) due to the flat nature of the substrate the reaction surface is at all times practically constant, one would expect according to Northrop (3) an approximation to this monomolecular reaction.

*Determination and Calculation of Unknown Solutions.*—The results obtained when films are digested with varying dilutions of a pepsin solution of undetermined proteolytic activity, although accurate, are merely comparative. As has been stated, films will vary in their initial readings. Also the silver suspension, by means of which the light intensity is measured, will vary with the films. It is impossible, therefore, directly to measure an unknown solution and express the results in absolute units because the standard for comparison is not constant. An unknown peptic activity can only be determined by comparing it with solutions of known digestive power run under the same conditions of test. In this way accurate determinations of proteolytic activity of the unknown solutions can be made and directly compared with that of a known standard. Varying dilutions of Armour's commercial pepsin 1:10,000 are used as a standard. All solutions are adjusted to the same pH by dilution with a buffer solution and are run in a manner similar to those illustrated in Fig. 1, the concentration of enzyme being the variable. At the same time, with films of the same initial reading, and with the same silver suspen-

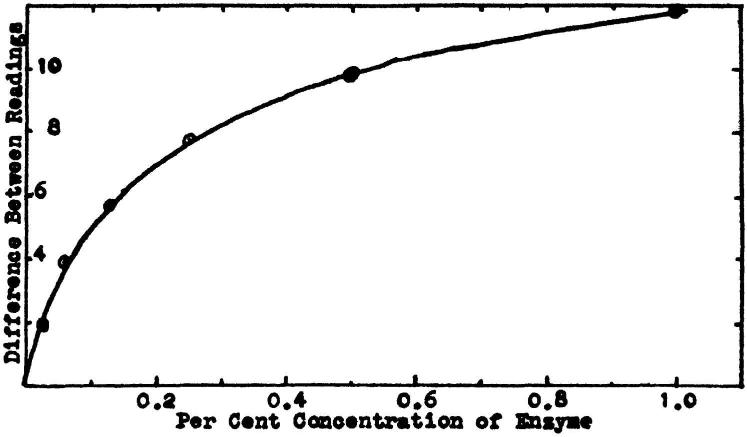

Fig. 1. Change in opacity of films due to peptic digestion. The differences on the abscissæ are expressed in mm.

sion to measure all the films, digestions of the unknown samples are run. The digestion curve is plotted from the values obtained from the known solutions. The values of the unknown solutions are then interpolated from this curve and are expressed as per cent of the known solutions.

As illustration, the determinations of two samples of gastric juice, secreted in response to a toast and tea test meal are shown. These samples vary appreciably in peptic activity. Six dilutions of Armour's commercial pepsin 1:10,000 ranging from a 1.0 to 0.03 per cent serve as the standards. The digestion curve obtained from the readings of these standards is shown in Fig. 3, where enzyme concentrations are plotted on the logarithmic scale of semilogarithmic paper. The concentrations of the unknown samples may thus be read directly.

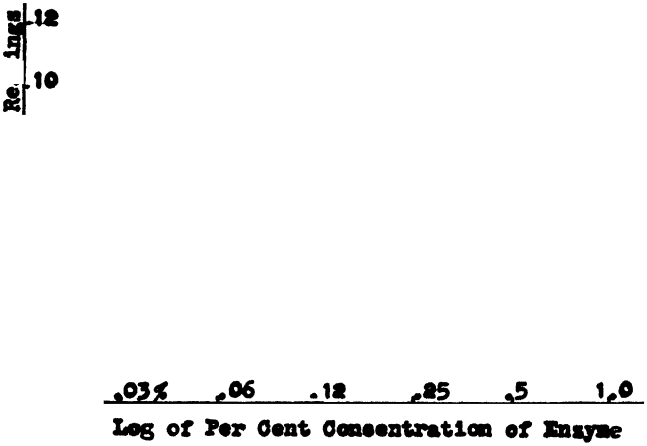

FIG. 2. Change in opacity of films due to peptic digestion. The differences on the abscissæ are expressed in mm.

As can be seen from Fig. 3, a 50 per cent dilution of unknown Sample 1 was equal in strength to a 0.26 per cent solution of standard 1:10,000 pepsin. Thus the concentration of enzyme in the undiluted sample is equivalent to the concentration of enzyme in a 0.52 per cent solution of 1:10,000 pepsin. Expressing the pepsin value of a 1.0 per cent solution of 1:10,000 pepsin optionally as 1,000 units, then we can express the concentration of the unknown as 520 units.

In a similar manner a 50 per cent dilution of Sample 2 is equivalent in proteolytic activity to a 0.116 per cent 1:10,000 pepsin solution. The undiluted sample is equal in strength to a 0.232 per cent solution, and the concentration of pepsin is 232 units. Thus proteolytic activity is expressed in very comprehensive terms; namely, as a definite concentration of an accurate and easily reproduced standard enzyme solution.

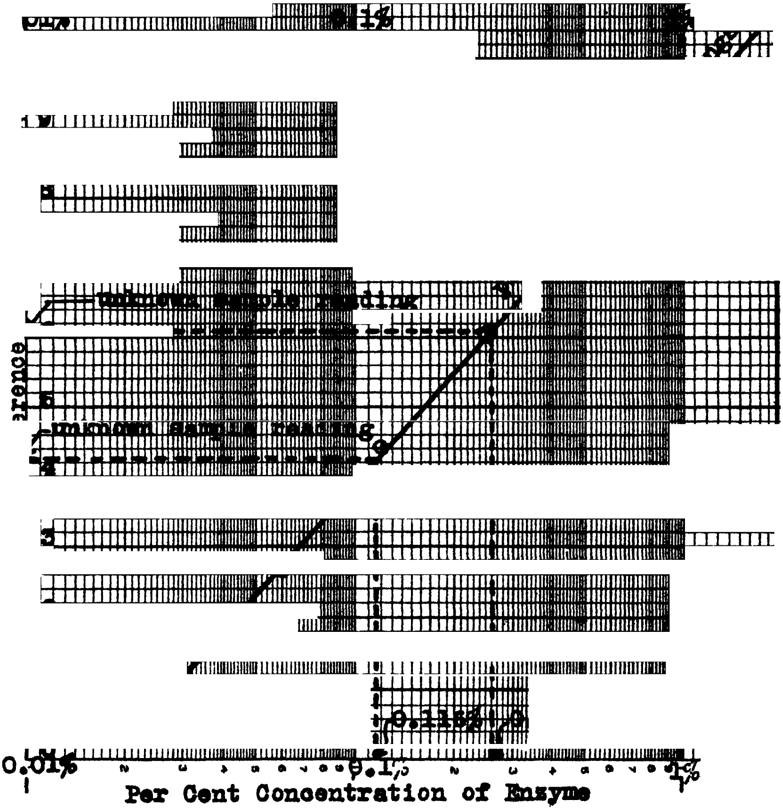

Fig. 3. Change in opacity of films due to peptic digestion.

*Preparation of Standard Solutions.*—The standard solution of 1.0 per cent strength is made at frequent intervals, 0.25 gm. of dry commercial pepsin being dissolved in 25 cc. of a buffer solution and thus brought to the optimal pH. Other dilutions are made from this 1 per cent solution. Inasmuch as only 1 cc. of the standard solution is necessary to make an entire series of varying dilutions, this 25 cc. will serve as a standard for about a month, during which time there is no demonstrable loss of activity if kept in the cold. It is estimated that a 4 ounce bottle of commercial pepsin will furnish material for a referable standard for many years. Armour's commercial pepsin has been found over a period of 2 years to lose not over 10 per cent of its digestive power (4). All determinations over a long period of time can thus be made referable to the same standard and accurate comparative values obtained.

## DISCUSSION.

All measurements of proteolytic activity involve either (1) an estimation by chemical or physical means of the amount of substrate digested per unit time or (2) a measurement of the time required to digest a unit of substrate. In either case, however, the accuracy of the method is dependent upon the accuracy with which one can measure the amount of protein that disappears or the unaffected residue. In the case of the method here developed for the quantitative estimation of peptic activity, the amount of substrate digested is so slight that, if the extent of hydrolysis were to be measured by any of the common chemical methods, it would be inappreciable. Yet with the digestion of this minute amount of substrate there is a change from opacity to transparency, permitting a wide range of differential readings. This extreme sensitivity in measuring proteolysis makes Gates' method valuable in the quantitative estimation of extremely dilute enzyme solutions, and also in the qualitative demonstration of the slightest proteolytic activity. Thus proteolytic activity, that might normally escape detection by methods in common use, can be accurately measured.

The time-saving element of the method is another of its valuable and important features. Cells can easily be assembled and placed in the water bath at 1 minute intervals. A 50 per cent dilution of normal gastric juice will give the optimal amount of digestion in 10 minutes. Thus thirty films can be digested in the course of 1 hour. By running triplicate checks, ten unknowns can be determined in 1 hour's time plus the time required to run the six standards and for the reading of the films.

The time that the films should be allowed to digest varies with the samples. The digestions should proceed until the films are from 25 to 50 per cent digested. This allows greatest accuracy in the reading of the films and at the same time gives results that fall on the steepest portion of the digestion curve illustrated by Fig. 1. However the digestion periods should be at least 10 minutes in length so that no appreciable error due to variation in timing results. Therefore, in the determination of solutions having a strong proteolytic activity, these solutions should be diluted until the digestion is optimal in 10 minutes. This can be ascertained in a preliminary digestion series in which the concentration of the unknown is the variable. In this preliminary trial, after a little experience one can easily estimate without the accuracy of instrument reading the dilutions at which to run the digestions. In the determination of a more dilute sample the digestion period is proportionally lengthened. At the same time a more dilute series of standards is used. With experience one learns just what dilution of standards to run with various digestion periods so as to run the gamut from opacity to transparency of the standard films and thus plot an accurate standard digestion curve.

Lastly, this method has the feature of requiring only a very minute volume of sample for a determination. The capacity of the digestion chamber is 0.5 cc. All samples are diluted at least to half strength so that the maximum volume required for duplicate determinations is 0.5 cc. of the original solution to be tested. In the case of more concentrated enzyme solutions, the required amount is less, inasmuch as the dilution of the sample must be greater. This is of great value in fractional analysis of gastric samples where a greater portion of the sample is used for the determination of other constituents.

Although all references in this paper are to the procedure for the determination of peptic activity, the method is equally applicable to the determination of proteolytic enzymes acting in alkaline medium. The only essential change that must be made concerns the buffer solution employed for dilution of the samples.

## SUMMARY.

1. A technique for the quantitative estimation of gastric proteolytic activity based upon the Gates method is presented.

2. The accuracy, advantages, and applications of this technique are discussed.

The authors wish to express to Dr. Frederick L. Gates their appreciation of his thoughtful criticisms of the manuscript of this paper; they also appreciate his generosity in consenting to publication of it before supplementing his preliminary paper by a more extended report of his own work.

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# INTESTINAL NUCLEOTIDASE.

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## INTRODUCTION.

The necessity of using enzymatic hydrolysis of the nucleic acids for the study of their structure has long been realized and earlier work towards this end led to the discovery of enzymes[1] capable of splitting nucleic acid to nucleotides, to nucleosides, or to phosphosugars. In recent years[2] these enzymes have been the object of considerable attention, particularly those classed as nucleotidases (phosphatases) which bring about the cleavage of phosphoric acid from the molecules. This paper deals with this latter type of enzymes which occur in the intestinal secretions.

Nucleotidases are widely distributed in the animal body. However, they are accompanied by other enzymes of which the nucleosidases are the most disturbing inasmuch as such preparations carry the hydrolysis beyond the phase of nucleosides. Fortunately, the intestinal secretion is free of nucleosidases. By virtue of this

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[1] Levene, P. A., and Medigreceanu, F., *J. Biol. Chem.*, **9**, 65, 375, 389 (1911).

[2] See for example: Levene, P. A., Yamagawa, M., and Weber, I., *J. Biol. Chem.*, **60**, 693 (1924). Levene, P. A., and Weber, I., *J. Biol. Chem.*, **60**, 707, 717 (1924). von Euler, H., and Brunius, E., *Ark. Kemi Mineral. Geol.*, **9**, 1 (1927); *Ber. chem. Ges.*, **60**, 1584 (1927). Martland, M., and Robison, R., *Biochem. J.*, **21**, 665 (1927); **23**, 237 (1929). Deutsch, W., *Z. physiol. Chem.*, **171**, 266 (1927). Erdtmann, H., *Z. physiol. Chem.*, **172**, 182 (1927); **177**, 211, 231 (1928). Akasawa, *J. Biochem.*, Japan, **10**, 157 (1928). Levene, P. A., and London, E. S., *J. Biol. Chem.*, **81**, 711 (1929); **83**, 793 (1929). Hommerberg, C., *Z. physiol. Chem.*, **185**, 123 (1929). Deutsch, W., and Rösler, K., *Z. physiol. Chem.*, **185**, 146 (1929). Deutsch, W., and Laser, R., *Z. physiol. Chem.*, **186**, 1 (1929). Tannhauser, S. J., and Angermann, M., *Z. physiol. Chem.*, **186**, 13 (1929).

property, intestinal juice was used successfully for the preparation of nucleosides of the thymus nucleic acid.[3]

Thus a nucleotidase free of nucleosidase can be prepared; not however, a nucleosidase free of nucleotidase, and such an enzyme is needed for the preparation of ribophosphoric and of desoxyribophosphoric acids.

With a view of accomplishing the separation of the two enzymes a systematic study of the properties of nucleotidase was undertaken. The present paper deals with the question of the specificity of intestinal nucleotidase. In order to study this specificity, a comparative study was made of the action of the enzyme on a series of phosphoric esters as follows:

1. Glycerol phosphate (used as a standard for enzyme activity).
2. Phospho-sugars.
    - (*a*) Hexosemonophosphate from α-diacetonefructose.
    - (*b*) " " β-diacetonefructose.
    - (*c*) Neuberg monophosphate.
    - (*d*) Hexosediphosphate.
    - (*e*) " methylglucoside.
    - (*f*) Hexosemonophosphate from monoacetoneglucose.
    - (*g*) " " diacetoneglucose.
3. Nucleotides.
    - (*a*) Adenilic acid.
    - (*b*) Inosinic "
    - (*c*) Uridine phosphoric acid.
4. Nucleic acids.
    - (*a*) Yeast nucleic acid.
    - (*b*) Thymus nucleic acid.

The substrate concentrations were in general 0.161 molal in order to permit the isolation of the products of hydrolysis if desired. However, in the case of thymus nucleic acid this condition was a handicap inasmuch as the substrate or some hydrolytic product inhibits the reaction considerably at this concentration.

The hydrolyses were carried out at a pH of 8.6 to 8.7, this value being slightly below the optimum pH of 9.0 to 9.2. Since the dissociation constants of phosphoric acid, glycerol phosphate, and hexosephosphates are nearly the same[4] there was no change in pH

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[3] Levene, P. A., and London, E. S., *J. Biol. Chem.*, **81**, 711 (1929); **83**, 793 (1929).

[4] Meyerhoff, O., *Naturwissenschaften*, **14**, 1277 (1926).

on hydrolysis[5] and hence for these substrates no buffers were necessary. Likewise, none was required for the nucleotides, but for the nucleic acids suitable buffers, namely diethylbarbiturate, borate, and acetate, were used.

The initial rates of hydrolysis of all of the substances studied are given in Table I. For purposes of comparison, the rates in this table were calculated to correspond to an arbitrary enzyme concentration of 2 activity units[6] per cc. of substrate solution.

TABLE I.

*Comparative Rates of Hydrolysis.*

| Sample No. | Substance. | Initial rate, mg. P per cc. per hr. | Relative rate. |
|---|---|---|---|
| 1 | Glycerol phosphate | 2 20 | 1.00 |
| 2 b | Hexosemonophosphate from β-diacetonefructose | 2.20 | 1.00 |
| 2 e | Hexosediphosphate methylglucoside | 2.00 | 0.91 |
| 2 d | Hexosediphosphate | 1.96 | 0.89 |
| 2 f | Hexosemonophosphate from monoacetoneglucose | 1.94 | 0.88 |
| 2 c | Neuberg monophosphate | 1.90 | 0.86 |
| 2 g | Hexosemonophosphate from diacetoneglucose | 1 54 | 0.69 |
| 2 a | " " α-diacetonefructose | 1.40 | 0.64 |
| 3 c | Uridine phosphoric acid | 1.78 | 0.81 |
| 3 a | Adenilic acid | 1.30 | 0.59 |
| 3 b | Inosinic " | 0.80 | 0.36 |
| 4 a | Yeast nucleic acid | 0.15 | 0.07 |
| 4 b | Thymus " " | 0.10 | 0.05 |

The hydrolyses were nearly complete in 6 to 12 hours except in the case of the nucleic acids. Complete hydrolysis of the latter

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[5] When 0.016 molal glycerol phosphate (*i.e.*, 0.1 the usual concentrations) was used, a slight increase in pH was observed (∽ 0.2 to 0.3 pH) which is in agreement with the results published recently by Hommerberg. (Hommerberg, C., *Z. physiol. Chem.*, **185**, 123 (1929)). This author used exceedingly dilute solutions of glycerol phosphate (0.001 to 0.007 molal) and he found that the pH increased by nearly 1.0 unit during hydrolysis. The increase, however, cannot be due to the reason ascribed to it by this author, namely to the liberation of free phosphoric acid, for the reason indicated above.

[6] For definition, see p. 758.

compounds could not be accomplished even by adding additional portions of enzyme. It is interesting to note that the nucleotides hydrolyze nearly as rapidly as the sugar esters but that the nucleic acids hydrolyze much more slowly. Uridine phosphoric acid is more rapidly split than adenilic acid and this latter in turn more readily than inosinic acid. This order is different from that in the case of hydrolysis with acid, where adenilic acid is split more rapidly than either uridine phosphoric acid and inosinic acid (the rates of the last two being nearly the same). It is also seen that with regard to the intestinal enzyme, thymus nucleic acid is somewhat more resistant than yeast nucleic acid. The rates of hydrolysis of hexosediphosphate and hexosediphosphate methylglucoside are exactly the same. No reducing sugars could be detected in the hydrolysates of hexosediphosphate methylglucoside or of the nucleotides, as was expected because of the absence of nucleosidases[7] in the enzyme preparations used.

Instead of using the intestinal juice directly as in a previous work[3] the enzyme precipitated from its solutions with acetone was used. Different samples thus obtained hydrolyzed a standard glycerol phosphate solution at approximately the same rate. In addition, these samples showed a constant parallelism in their capacity for hydrolyzing both nucleotides and thymus nucleic acid. However, one sample showed an exceptional behavior. This sample had an activity towards glycerol phosphate slightly higher than the average and likewise hydrolyzed adenilic acid with the same high rate but hydrolyzed nucleic acid to phosphoric acid and to nucleosides very slowly, the initial rate being only one-tenth of the usual value. After 24 hours the extent of the hydrolysis was 17 per cent instead of 60 per cent. This sample had a distinctly green color instead of the customary yellow one, thus indicating the presence of bile products to which possibly the peculiar behavior of this sample may be attributed. It is equally conceivable that only slight hydrolysis occurred because of the absence of nucleinase, an enzyme converting polynucleotides into the component mononucleotides. If the latter explanation is correct, it may be assumed further that degradation of nucleic acids to nucleotides must precede the dephosphorylation, that is, the phosphatase is

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[7] Levene, P. A., and Medigreceanu, F., *J. Biol. Chem.*, **9**, 375 (1911).

capable of dephosphorylating nucleotides only, and not nucleic acids. This suggestion has already been made. Indeed, it was found that thymus nucleic acid was split more rapidly to nucleotides with the normal enzyme than with the abnormal sample.

On this assumption it is unnecessary to postulate the existence of more than one phosphatase.

The addition of magnesium ion in order to remove the inhibition by the phosphate ion liberated in the hydrolysis of thymus nucleic acid gave results that were not as satisfactory as those carried out in the absence of magnesium ion. It may be that the inhibition by the magnesium ion and the ammonium acetate outweighs the advantage gained by the removal of the phosphate. The hydrolysis curve, as will be noted, is quite unlike the usual curves but the per cent hydrolysis finally reached the normal value.

## EXPERIMENTAL.

*A. Standard of Phosphatase Activity.*—As a basis for the assay of the enzyme activity of the samples the hydrolysis of a standard sodium glycerol phosphate solution was used. This substrate is very convenient for this purpose since the hydrolysis may be readily followed directly on the hydrolysate.

This glycerol phosphatase activity is probably a true measure of the *general phosphatase activity* of the samples, since the capacity of the enzyme for hydrolyzing the different types of organic phosphates (such as the phospho-sugars and nucleotides, but with the exception of the nucleic acids) seems to be proportional to their capacity for hydrolyzing glycerol phosphate.

The glycerol phosphate standard solution was prepared by dissolving crystalline sodium glycerol phosphate (Eastman Kodak Company) in distilled water, the solution being made 0.322 molal on the basis of the phosphorus in the crystalline material. The solution was brought to a pH of 8.6, was protected with toluene, and was kept in the cold. The procedure in the assay was as follows: to 10.0 mg. of enzyme were added 2.5 cc. of water and, after thorough shaking, 2.5 cc. of this standard glycerol phosphate solution. The solution was placed in a thermostat at 30°, and phosphate ion determinations were made at convenient time intervals according to the method indicated later for the phosphosugars.

It was found convenient to adopt as a unit of activity the amount of enzyme capable of liberating from the glycerol phosphate substrate, 1.0 mg. of phosphorus (as phosphate ion) in the course of the 1st hour of the hydrolysis. This amount of phosphorus corresponds to a hydrolysis of 20 per cent since a 0.161 molal solution contains 5.0 mg. of phosphorus per cc. The activity coefficients are then given by dividing this initial rate by the weight of solid enzyme expressed in mg. per cc.

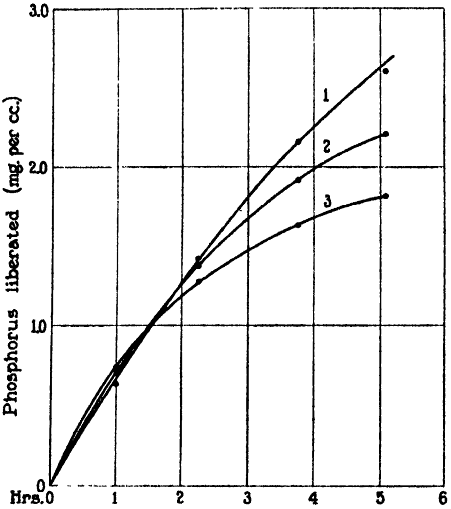

FIG. 1. Effect of glycerol phosphate concentration. Enzyme concentration = 1.0 mg. per cc. The glycerol phosphate concentration was for Curve 1, 0.322 M; Curve 2, 0.161 M; Curve 3, 0.080 M.

Within a limited range of concentrations the initial rates of hydrolysis are independent of the substrate concentration (Fig. 1) but are proportional to the enzyme concentration (Fig. 2).

*B. The Enzyme.*—The intestinal secretions of dogs collected from an intestinal fistula were the source of the crude enzyme material used. These solutions varied considerably as to amount, activity, and opacity. The solutions as obtained had a pH of 7.0 and were usually light yellow in color, though in some cases were distinctly green due perhaps to bile products. The solutions were filtered through cheese-cloth, centrifuged, and then poured into 10 volumes of acetone. The precipitate was quickly centrifuged, washed once with acetone, twice with anhydrous ether, and put into a vacuum desiccator to dry over phosphorus pentoxide and paraffin. The amount of precipitate obtainable in this way ranged from 5 to 10 mg. per cc. of solution but could be practically doubled by not centifuging so quickly after the precipitation. The material obtained by more complete precipitation was not so satisfactory since it was not as stable nor as active as that obtained by more rapid work. The crude dry enzymes thus obtained were fluffy powders and possessed the characteristic color of the solution from

FIG. 2. Effect of enzyme concentration. Glycerol phosphate concentration = 0.161 M.

| Enzyme concentration. | Initial rate of hydrolysis. |
|---|---|
| Curve 1, 2.0 mg. per cc. | 1.40 mg. P per cc. per hr. |
| " 2, 1.0 " " " | 0.69 " " " " " " |
| " 3, 0.5 " " " | 0.35 " " " " " " |

which they were precipitated except in a few cases where light yellow solutions yielded almost white samples. They were in general very stable and usually had activity coefficients between 0.6 and 0.8, though occasionally samples as high as 1.0 and as low as 0.5 were obtained. The dry enzyme samples had an inorganic phosphate content equivalent to about 3 per cent of phosphorus, there evidently being no organic phosphates present since no liberation of phosphorus was observed when they were allowed to stand in solution. It may be mentioned here that the original enzyme solutions when kept at 0° and protected with a small amount of toluene, also maintained their glycerol phosphatase activity unchanged for as long as a month. However, the character, amount, and activity of the precipitate prepared from solutions which were allowed to stand for some time, did not appear to be as satisfactory as those prepared from fresh enzyme solutions.

TABLE II.

*Enzyme Activities.*

| Material. | Activity units. | Activity coefficient (units per mg.). | Total activity recovered per cc of solution. |
|---|---|---|---|
| Original enzyme solution; dry weight, 23.7 mg. per cc | 1.45 per cc. | 0.061 | (1.45) |
| Acetone-precipitated enzyme | | | |
| (*a*) Usual method of precipitation. Yield 5.84 mg. per cc | 1.96 per 2 mg. | 0.98 | 5.72 |
| (*b*) Maximum precipitation. Yield 11.1 mg. per cc. | 1.00 per 2 mg. | 0.50 | 5.55 |

By the simple acetone precipitation considerable purification of the enzyme was obtained, the activity coefficient increasing about 15-fold. This is shown in Table II. It also shows the effect of the method of purification adopted as compared to that of more complete precipitation. Thus, by approximately doubling the amount of precipitate the activity coefficient was halved, hence the total activity recovered was not increased. The fact that there was a greater activity in the precipitates than was apparently present in the original solution is probably due to inhibiting substances contained in the latter.

*C. Methods. Hydrolyses and Analyses.*—The solutions of sodium salts of the phosphate substrates were prepared differently according to the form in which the material was available. The origin of these samples will be indicated under the separate hydrolysis experiments. In the case of sodium salts, they were dissolved directly in distilled water to the correct volume and adjusted to the desired pH. In the case of the free phosphoric acid esters they were dissolved in dilute alkali to the correct volume and pH. In the case of the barium salts they were added to water, the calculated quantity of sodium carbonate solution was added slowly with stirring, the solutions centrifuged, and the filtrates used after testing for the absence of barium with a trace of sulfate, and bringing them to the correct pH. The concentrations were usually such that on complete hydrolysis there would be liberated 5.0 mg. of phosphorus per cc. of hydrolysate, that is 0.161 molal (diphosphates, 0.080 molal). Values differing from 5.0 mg. per cc. are indicated in the legends of the figures. Such values are placed in parentheses following the name of the substrate. In the case of thymus nucleic acid it was found better to use one-third of this concentration, though solutions containing up to 6 per cent of this nucleic acid were sometimes used.

The enzyme concentrations were 2 mg. per cc. of substrate except in the case of the nucleic acids where higher and variable amounts (from 9 to 15 mg. per cc.) were used. Weighed amounts of enzyme were added directly to definite volumes of the substrates.

The hydrolyses were carried out in a thermostat maintained at 30°. The samples were in all cases protected from bacterial infection by a small amount of toluene, a substance found to have no effect on the hydrolysis rates. Both chloroform and sodium arsenate showed an inhibition. In some cases octyl alcohol was found useful as an antifroth in preparing the substrate solutions. It was interesting to find that it had a slight enhancing effect on the enzyme. This was indicated by the fact that the hydrolysis of glycerol phosphate in the presence of a few drops of this material was slightly more rapid than in its absence. The substrates were hydrolyzed at a pH of 8.6 to 8.7 and in most cases they maintained this value throughout the complete hydrolysis by virtue of their own buffering capacity at this pH. For the exception to this, the nucleic acids, buffers of diethylbarbiturate, borate, and acetate were used.[8] The buffer solutions were used in the hydrolyses in the ratio of 1 cc. of buffer to 2 cc. of the nucleic acid substrate solution. The buffering capacity even under these conditions was not quite sufficient to prevent completely a change in the pH during the hydrolysis; small amounts of alkali were added occasionally, in order to maintain the pH within the range of 8.3 to 8.7.

The rate measurements were made by determining colorimetrically the free phosphate ion concentration. The method of Kuttner and Cohen[9] was used. Nearly all of the experiments were made in duplicate. Initial determinations of the phosphate were always made and this value which was quite variable, though small, was corrected for in the curves which are given. Suitable samples were removed at convenient time intervals from the hydrolysates and treated differently according to the type of substrate. For the phospho-sugars, 0.5 cc. of the hydrolysate was pipetted into 4.5 cc. of 1 per cent trichloroacetic acid which was then filtered with the aid of a small amount of kieselguhr through an ordinary filter. This solution was diluted so as to be approximately equivalent to the standard containing 0.025 mg. of phosphorus per cc. 1 cc. samples were then compared with the standard. In the case of the nucleotides and the nucleic acids the procedure was necessarily modified since the determinations could not be made directly on the trichloroacetic acid filtrates. The nucleotides did not precipitate with the trichloroacetic acid and interfered in the colorimetric method. The nucleic acid filtrates could not be clarified nor could the nucleic acid be completely precipitated. This later caused interference. For the nucleotides 0.5 cc. samples of the hydrolysates were added to 2 cc. of water and 1 cc. of magnesia mixture.[10] To this, a drop of dilute ammonia was

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[8] The veronal buffer of pH 8.6 was made according to the directions of Michaelis (Michaelis, L., *J. Biol. Chem.*, **87**, 33 (1930)). The borate buffer was the standard Clark and Lubs' (Clark, W. M., The determination of hydrogen ions, Baltimore (1928)) boric acid-potassium chloride-sodium hydroxide buffer of pH 8.6. The acetate buffer was prepared by dissolving 38.5 gm. (0.5 mol) of ammonium acetate in 13.5 cc. of 5 N ammonium hydroxide and diluting to 500 cc.

[9] Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, **75**, 517 (1927).

[10] 1 liter of the magnesia mixture used contained 75 gm. of magnesium chloride (6 mols of water) and 100 gm. of ammonium chloride.

immediately added and the solution stirred thoroughly. The magnesium ammonium phosphate precipitated in this way was always crystalline. A minute later 1 cc. of concentrated ammonium hydroxide (sp. gr. 0.90) was added. Because the enzymatic hydrolysis continued with an appreciable rate even in these strong ammonia solutions the precipitates were allowed to stand only 10 to 15 minutes, after which time they were filtered through a micro filter and washed with 2 cc. of 2 per cent ammonium hydroxide. The precipitates were redissolved in 10 cc. of 0.5 N HCl, made up to 25 cc., diluted if necessary, and determined as usual. Actually, under the conditions employed the precipitation of the phosphate is essentially complete even in this short interval of time.

In the nucleic acid experiments prior to precipitating the phosphate the unchanged nucleic acid was removed. This was accomplished by pipetting 5 cc. samples of the hydrolysate into an equal volume of 10 per cent trichloroacetic acid, adding some kieselguhr, and filtering with suction. The filtrate, which was still turbid, became nearly clear when ammonia was added until slightly alkaline. After a short time the remaining turbid material coagulated and could be removed easily by filtering with an ordinary filter. To the filtrate (about 70 cc.) 3 to 5 cc. of magnesia mixture were added and the precipitate allowed to become well formed before 12 cc. of concentrated ammonia were added. The precipitate was allowed to stand overnight, was filtered off in a small Gooch funnel, dissolved in 15 cc. of 1 N HCl, and the phosphate determination performed as usual.

In order to avoid the inhibition effect of the phosphate[11] it was desirable to carry out a hydrolysis in which the phosphate was removed as fast as formed. The method was similar to that reported.[12] The nucleic acid was dissolved in magnesia mixture[13] instead of in water, the solution was allowed to stand overnight,

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[11] See Hommerberg, C., *Z. physiol. Chem.*, **185**, 130 (1929).

[12] Deutsch, W., *Z. physiol. Chem.*, **186**, 11 (1929). The amount of magnesium ion used in the hydrolysis experiment recorded there is obviously insufficient for the precipitation of the phosphate at the end of the hydrolysis.

[13] 1 liter of the magnesia mixture used contained 57 gm. of magnesium acetate (4 mols of water), 180 gm. of ammonium acetate, and 60 cc. of a 10 per cent ammonium hydroxide solution. The solution was 0.25 molal in respect to $Mg^{++}$ and had a pH of 8.6.

and filtered. The filtrate was used with the acetate buffer in the usual manner. The phosphate which precipitated during the reaction was readily dissolved by 10 per cent trichloroacetic acid, so no modification of the phosphate analysis was necessary.

*D. Hydrolysis of Phospho-Sugars.*—The barium salts of the phosphoric acid esters from α- and β-diacetonefructose are those previously prepared.[14] The barium salt of the Neuberg ester was prepared according to the method of Neuberg.[15] The substrates for the hydrolyses were made from these samples as indicated above. The hydrolysis curves are shown in Fig. 3. The ester from β-diacetonefructose hydrolyzed at exactly the same rate as glycerol phosphate, whereas the ester from α-diacetonefructose

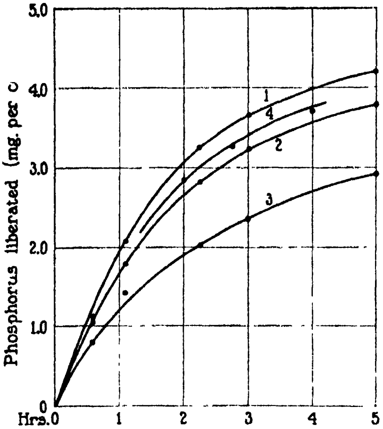

Fig. 3.

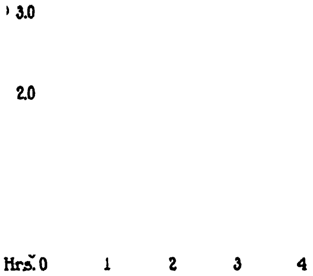

Fig. 4.

Fig. 3. Hydrolysis of fructose phosphoric esters. Curve 1, glycerol phosphate standard; hexosemonophosphate from β-diacetonefructose; Curve 2, Neuberg monophosphate; Curve 3, hexosemonophosphate from α-diacetonefructose; Curve 4, hexosediphosphate and the corresponding methylglucoside.

Fig. 4. Hydrolysis of glucose phosphoric esters. Curve 1, hexosemonophosphate from monoacetoneglucose (4.5 mg. P per cc.); Curve 2, hexosemonophosphate from diacetoneglucose; Curve 3, glycerol phosphate standard.

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[14] Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **83**, 619 (1929).

[15] Neuberg, C., *Biochem. Z.*, **88**, 432 (1918).

hydrolyzed more slowly. The Neuberg monophosphate has a hydrolysis rate approaching that of the ester from β-diacetonefructose. Thus the rate of hydrolysis of a phospho-sugar varies greatly with the position occupied by the phosphoric acid radical.

The hexosediphosphate barium salt was prepared from the commercial calcium salt through the intermediary of the sodium salt. The methylglucoside of this hexosediphosphate obtained as the barium salt is that previously described.[16] The hydrolysis curves for the two substances were practically identical and were midway between the curves (Fig. 3) for the ester from β-diacetonefructose and the Neuberg ester.

The hexosemonophosphates from monoacetone-[17] and diacetoneglucose[14] were isolated as barium salts after having been purified as brucine salts. The hydrolysis curves of the substrates prepared from these samples are given in Fig. 4. The monophosphate obtained from monoacetoneglucose hydrolyzed at the same rate as the Neuberg ester. The rate of hydrolysis of the monophosphate from diacetoneglucose was lower than that of the preceding monophosphate, its rate being approximately the same as that of the phosphoric acid ester from α-diacetonefructose.

*E. Hydrolysis of Nucleotides.*—Two purine derivatives, adenilic acid and inosinic acid were hydrolyzed. The former was obtained as the free crystalline acid and the latter as the barium salt. The pyrimidine nucleotide, uridine phosphoric acid, was obtained as the barium salt. The hydrolysis curves of the substrates prepared from these samples are given in Fig. 5. The pyrimidine nucleotide, uridine phosphoric acid, is more rapidly split than the purine nucleotides, its rate being slightly lower than that of the Neuberg ester. Of the two purine nucleotides, adenilic acid is more rapidly split than inosinic acid, the rate of the first being the same and that of the second being lower than that of the phosphoric acid ester from α-diacetonefructose.

For a comparison, the hydrolysis curve of yeast nucleic acid is plotted in Fig. 5 with those of the nucleotides. The comparison is only qualitative since the enzyme concentration in the hydrolysis of the nucleic acid was 4.5 times that used in the case of

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[16] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **80**, 633 (1928).

[17] Prepared by phosphorylation of monoacetoneglucose and subsequent hydrolysis.

nucleotides. The contrast between the rates of hydrolysis of the nucleotides and the nucleic acids is indicated more strikingly in Table I, where the comparative rates are given for a standard amount of enzyme.

*F. Hydrolysis of Nucleic Acids.*—Yeast nucleic acid, as the free acid, and thymus nucleic acid, as the semi-crude sodium salt and as purified free acid, were used as substrates. Various curves of the hydrolysis of these materials are given in Figs. 6 to 10.

In Fig. 6 is shown a comparison of the rates and the extents of hydrolysis of yeast and thymus nucleic acids. The former hydrolyzes faster and reaches a greater per cent hydrolysis than does

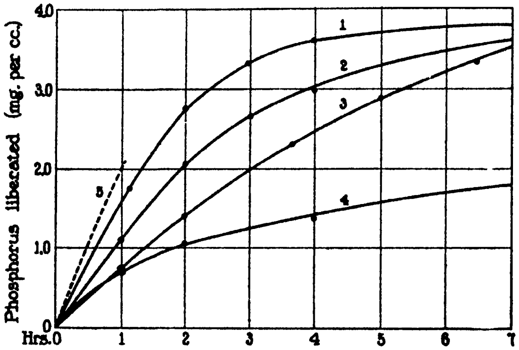


Fig. 5. Hydrolysis of nucleotides. Curve 1, uridine phosphoric acid (3.9 mg. P per cc.); Curve 2, adenilic acid (4.1 mg. P per cc.); Curve 3, inosinic acid; Curve 4, yeast nucleic acid; Curve 5, glycerol phosphate standard.

the latter, showing the thymus nucleic acid to be somewhat more stable than yeast nucleic acid, though the difference between the two does not appear to be as great as would be supposed from previous indications.[1,3]

The effect of the buffers on the rate of hydrolysis of thymus nucleic acid is given in Fig. 7. The acetate buffer inhibits the hydrolysis of the glycerol phosphate standard to the extent of about 20 per cent, whereas the other two buffers show no effect on this hydrolysis. It is probable that the acetate buffer also inhibits the nucleic acid hydrolysis by the same amount and taking this into account by using a higher concentration of enzyme with this buffer, exactly similar hydrolysis curves were obtained with the acetate and veronal buffers. However, the borate buffer had a peculiar effect on the nucleic acid hydrolysis, though it showed no effect on the hydrolysis of glycerol phosphate. For this buffer the hydrolysis curves showed a marked break between 15 to 20 per

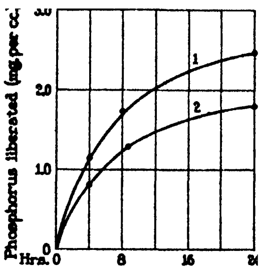


FIG. 6.

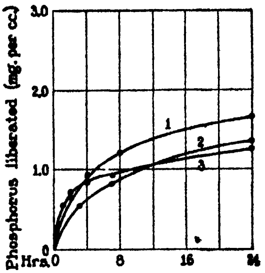


FIG. 7.

FIG. 6. Hydrolysis of yeast and thymus nucleic acids. Curve 1, yeast nucleic acid (5.0 mg. P per cc). Maximum value reached = 3.8 mg. P per cc.; Curve 2, thymus nucleic acid (4.0 mg. P per cc.). Maximum value reached = 2.5 mg. P per cc.

FIG. 7. Hydrolysis of thymus nucleic acid. Effect of buffers: Curve 1, with veronal or acetate buffer; Curve 2, with acetate buffer and Mg$^{++}$; Curve 3, with borate buffer.

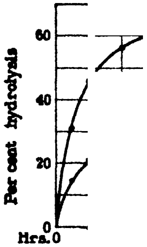


FIG. 8.

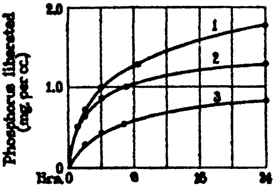


FIG. 9.

FIG. 8. Hydrolysis of thymus nucleic acid. Effect of substrate concentration: Curve 1, 2 per cent nucleic acid solution (1.3 mg. P per cc.); Curve 2, 6 per cent nucleic acid solution (4.0 mg. P per cc.).

FIG. 9. Hydrolysis of thymus nucleic acid. Effect of purification: Curve 1, unpurified and satisfactorily purified samples (4.0 mg. P per cc.); Curves 2 and 3, unsatisfactorily purified samples (4.0 mg. P per cc.).

cent hydrolysis though the rates were entirely normal up to this point. This effect is perhaps due to the combination of some partially hydrolyzed portion of the nucleic acid with boric acid. The decrease in hydrolysis rate brought about by the addition of magnesium ion to the reaction mixture is also indicated in Fig. 7.

In the hydrolysis of the phospho-sugars and the nucleotides, the substrate concentrations were 0.161 molal. In these cases the hydrolysis was always complete after a sufficient length of time (12 to 24 hours). On the other hand the hydrolysis of the nucleic acids at this concentration could not be made complete even by adding additional portions of enzyme and allowing the hydrolysis to continue for a number of days. It was found advantageous to use a more dilute solution of these substrates since both the rate and the extent of hydrolysis could be materially increased as shown in Fig. 8, though complete hydrolysis was not accomplished even in these dilute solutions.

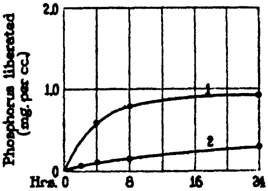


FIG. 10. Hydrolysis of thymus nucleic acid. Effect of enzyme sample: Curve 1, average sample—phosphatase activity = 1.7 (1.3 mg. P per cc.); Curve 2, abnormal sample—phosphatase activity = 2.0 (1.3 mg. P per cc.).

The preliminary hydrolyses of thymus nucleic acid were carried out on a semi-crude sample. Later it was desired to use better material but in several cases the purification of the thymus nucleic acid yielded samples which had much lower rates of hydrolysis than the unpurified sample. While the chemical analysis of these slowly hydrolyzed samples indicated that they were not as pure as desired, the differences from the correct analysis values were not as great as might be expected from the deviations noted in the rates of hydrolysis. These samples possibly contained some diphosphoric esters of pyrimidine nucleosides. Careful purification yielded samples which analyzed perfectly and hydrolyzed with the same rate as the unpurified sample. A comparison of these various samples can be made from Fig. 9.

The anomolous activity of the enzyme sample mentioned in the introduction is indicated in Fig. 10. Curve 1 represents the hydrolysis of thymus nucleic acid with a normal sample of the enzyme which, as indicated in the previous discussion, shows a high rate of hydrolysis with both glycerol phosphate and adenilic acid. Curve 2 represents the hydrolysis with the abnormal sample which also hydrolyzes glycerol phosphate and adenilic acid with the same high rate as the normal sample. A more striking comparison is obtained from Table III which gives the initial rates of hydrolysis of the three types of substrates with the two different enzyme samples.

TABLE III.

*Comparative Rates of Hydrolysis.*

| Substrate. | Initial rate of hydrolysis. | | | |
|---|---|---|---|---|
| | With normal sample. | | With abnormal sample. | |
| | Mg. P per cc. per hr. | Relative rate. | Mg. P per cc. per hr. | Relative rate. |
| Glycerol phosphate | 1.65 | 1.00 | 2.03 | 1.23 |
| Adenilic acid | 0.95 | 0.58 | 1.06 | 0.64 |
| Thymus nucleic acid | 0.25 | 0.16 | 0.022 | 0.013 |

# STUDIES IN POLYMERIZATION AND CONDENSATION.

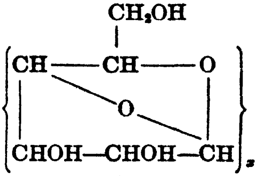
## VI. 5,6-DIHYDROXYHEXANONE-2.

By P. A. LEVENE and A. WALTI.

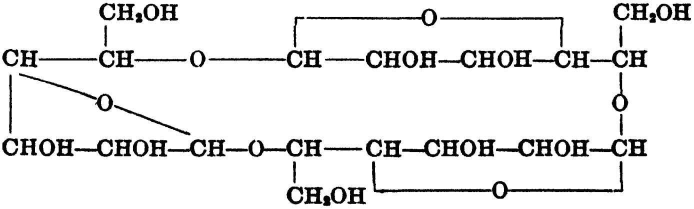
*(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)*

(Received for publication, July 15, 1930.)

In a previous communication[1] a report was made on the spontaneous condensation of dihydroxyacetone into dihydroxyacetonyldihydroxyacetone and di-(dihydroxyacetonyl)dihydroxyacetone. It seemed desirable to extend the observations to simple dihydroxyketones in which the positions of the two hydroxyls were on carbon atoms 5 and 6 for the reason that such an arrangement would permit <2, 5> and <2, 6> lactal formation and hence present conditions similar to those existing in common sugars. The investigation seemed all the more desirable for the reason that Hibbert and Timm[2] had already studied one condensation product of 5,6-dihydroxyhexanone-2 and had attributed to it a structure analogous to that which Hibbert had postulated for cellulose.



I.



II.

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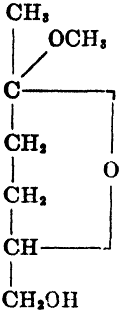
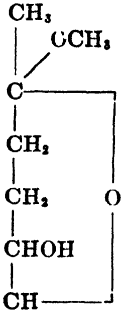
[1] Levene, P. A., and Walti, A., *J. Biol. Chem.*, **78**, 23 (1928).

[2] Hibbert, H., and Timm, J. A., *J. Am. Chem. Soc.*, **45**, 2433 (1923).

However, before entering into the study of the polymerization or condensation of 5,6-dihydroxyhexanone-2, it was desirable to obtain more information on the structure of the substance itself, particularly with respect to its cyclic nature. In the homogeneous state the substance probably consists of the two forms in equilibrium. In some of its derivatives, however, the cyclic form apparently predominates. Thus, on treatment with methyl alcohol containing 0.5 per cent of hydrogen chloride, a methylcyclo derivative is obtained, having one of the following structures.



III.

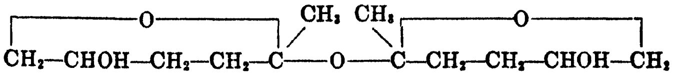


IV.

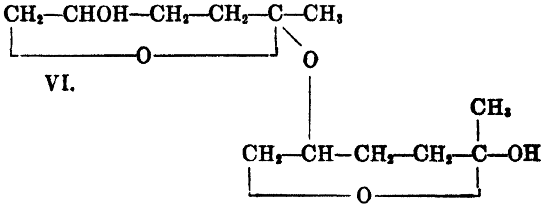
The molecular refraction of the substance is 36.97 and the calculated value is 37.13. In harmony with the view of the cyclic structure were also the results of further methylation and of acetylation inasmuch as the methylcyclo derivative gave a monoacetyl and a monomethyl derivative.

### *Condensation of 5,6-Dihydroxyhexanone-2.*

Freshly distilled ketone is a mobile colorless liquid. Often, on standing at 25° and sometimes even on standing at 0°, as much as 50 per cent of the original ketone undergoes condensation into a substance distilling at 175–180° at 0.2 to 0.3 mm. pressure and having the composition of a desoxydisaccharide, dihydroxyhexanonyldihydroxyhexanone. As such, the substance gave a diacetyl derivative. However, after treatment at room temperature with dry methyl alcohol containing 0.5 per cent of hydrogen chloride the disaccharide was apparently hydrolyzed with the formation of methylcyclodihydroxyhexanone-2. The great instability of the desoxydisaccharide is not surprizing in view of the fact that 5,6-dihydroxyhexanone-2 is a desoxy sugar similar to glucodesose and in view of our present knowledge of the great instability of the glucosides of this group of substances. Because of the great instability of this disaccharide, it is difficult to choose between the two structures V and VI which it may possess; however, structure V is the more probable one.



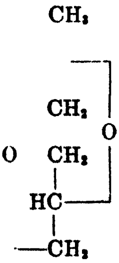
V.



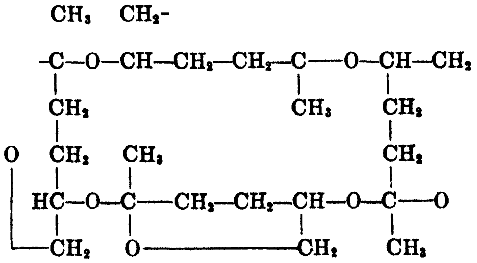
VI.

*Polymerization Product Obtained from 5,6-Dihydroxyhexanone-2 with Sulfuric Acid as Catalyst (Hibbert and Timm).*

In the light of the above result on the spontaneous formation of the disaccharide, it became necessary to reinvestigate the structure of the substance prepared by Hibbert and Timm[2] which according to these authors should have one of the following structures in analogy with the structure postulated by Hibbert for cellulose.



VII.

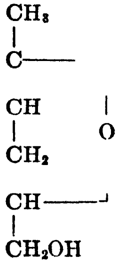


VIII.

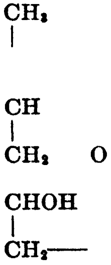
As it is seen from figures VII and VIII, substances of this structure have no free hydroxyls and therefore do not permit the formation of acetyl derivatives. In reality, however, the substance prepared by us according to Hibbert and Timm formed an acetyl derivative containing one acetyl group for each dihydroxyketone radical and yet the substance had the composition of an anhydrodihydroxyhexanone, in agreement with the analytical data of Hibbert and Timm. In order, therefore, to explain the structure of the substance of Hibbert and Timm, it was necessary to obtain more information on the structure of anhydro-5,6-dihydroxyhexanone-2.

### *Anhydride of 5,6-Dihydroxyhexanone-2.*

Theoretically the anhydride may be formed either through a second ring formation similar to that observed in the case of glucosans (structure VII) or through desaturation (structures IX or X).



IX.



X.

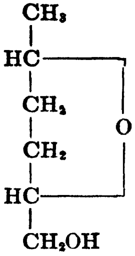
Indeed, the existence of the two forms (VII and IX or VII and X) could be easily demonstrated. On heating 5,6-dihydroxyhexanone-2 to 150° for 9 hours there occurred a loss of water. The lower boiling fraction of this product consists apparently of the anhydride of structure VII on the basis of the values of the molecular refraction and of the parachor. On the other hand, if the condensation product is acetylated prior to distillation and the acetylation product is distilled, then a substance is obtained with the composition of a monoacetyl derivative of the anhydrodihydroxyhexanone. Its structure is seen from the fact that it contains an acetyl group and furthermore from the fact that in the presence of Adams' catalyst, this substance absorbs hydrogen in an amount corresponding to one double bond. Also the determined values of the molecular refraction and of the parachor are in agreement with the calculated ones for the acetyl derivative of substances IX or X.

### *Polymerization of the Anhydro-5,6-Dihydroxyhexanone-2.*

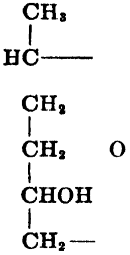
At times, a hard, slightly brownish resin was obtained on heating the 5,6-dihydroxyhexanone-2 at 150° for 10½ hours with interruptions after each 3½ hour period. This material on heating under reduced pressure and at a temperature not exceeding 230° lost only water and the residue had the composition of anhydrodihydroxyhexanone. On acetylation, this polymerized product formed an acetyl derivative which had the composition of a monoacetyl derivative of an anhydrodihydroxyhexanone, thus showing that the hydroxyl of the anhydride is not involved in the polymerization. On the other hand, on standing at room temperature in methyl alcohol containing 0.5 per cent of hydrogen chloride, the substance remained unchanged; on refluxing with the same solution, the substance was methylated only to the extent of one methyl group to about five of the ketone. It is not excluded that under these conditions a slight depolymerization took place. Also, the absorption of hydrogen in the presence of Adams' platinum dioxide catalyst was very small, approximately 1 molecule of the gas being absorbed for every 7 molecules of the anhydride. All these properties seem to indicate that the double bond is involved in the polymerization. Thus, it seems to us that the 5,6-dihydroxyhexanone-2 cannot serve as a model of a true sugar and its polymerization product cannot serve as a model of a polysaccharide. Rather is 5,6-dihydroxyhexanone-2 a model of a desoxy sugar and the resin formation which so readily takes place a model of the events in the 2-desoxy sugars. Indeed, the tendency of the latter to polymerize with tar formation is one of their principal characteristics. *It is possible that the mechanism of polymerization of desoxyglucoses is analogous to the mechanism of polymerization of 5,6-dihydroxyhexanone-2 which takes place in two steps; namely, anhydride formation giving rise to a double bond which then causes the new substance to polymerize. The same is undoubtedly the process of formation of the substance of Hibbert and Timm.*

### *Reduction of 5,6-Dihydroxyhexanone-2.*

In conclusion, an interesting reaction of the 5,6-dihydroxyhexanone-2 may be mentioned which in a way, may go to show the readiness with which the substance undergoes desaturation. When the dihydroxyketone in acetic acid solution is acted upon by hydrogen gas in the presence of Adams' platinum dioxide catalyst, the molecule loses 1 hydroxyl and the resulting substance forms on acetylation only a monoacetyl derivative. The most probable structure of the new substance is that of a tetrahydrofuran or of a tetrahydropyran derivative (XI or XII).

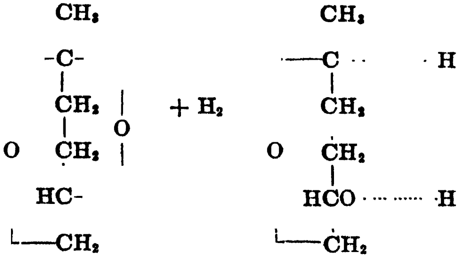


XI.



XII.

The formation of this substance from the original cycloketone may take place in three different ways. (*a*) It may be preceded by the formation of the anhydride IX or X. (*b*) It may be preceded by the formation of the anhydride VII, which undergoes reductive opening of one ring, 1 hydrogen atom being added to carbon atom 2, and the other to the oxygen atom involved in the ring formation.



XIII. XIV.

(*c*) The hydroxyl in position (2) of the cyclic ketone may undergo direct reduction. The last possibility does not seem to us probable in view of the fact that experiments in our laboratory have shown that tertiary aliphatic carbinols do not undergo reduction on treatment with hydrogen in the presence of Adams' catalyst. Of the other two reactions, the first one seems to us the more probable.

## EXPERIMENTAL.

### *5,6-Dihydroxyhexanone-2.*

5,6-Dihydroxyhexanone-2 was prepared from α-acetyl-δ-chloro-γ-valerolactone by the procedure of Traube and Lehmann[3] modified slightly, as follows: To the cooled solution of 47 gm. of sodium in 800 cc. of absolute alcohol 260 gm. of freshly distilled ethylacetoacetate were slowly added. To this cooled solution (temperature 25–30°) in turn were added 190 gm. of redistilled epichlorohydrin dissolved in 250 cc. of absolute alcohol in such a manner that the temperature did not rise above 35°. If the temperature was kept at zero degrees, a poor yield of acetylchlorolactone was obtained. The reaction mixture was continuously stirred. After allowing the mixture to stand overnight, it was concentrated under reduced pressure and the residue was treated with 2000 cc. of 5 per cent sulfuric acid in a separatory funnel. An oil separated which was dissolved in ether and dried over sodium sulfate. After removal of the solvent the lactone distilled at 110–112° at 0.2 mm. Yield 230 gm. (65 per cent).

Redistilled α-acetyl-δ-chloro-γ-valerolactone (214 gm.) was refluxed with a solution of 85 gm. (0.5 mol) of potassium carbonate in 1615 cc. of water for 4 hours. The oil gradually dissolved with evolution of carbon dioxide. The solution was concentrated at a low temperature under diminished pressure. The residue was dissolved in absolute alcohol, the solution filtered with suction from the potassium chloride, and then concentrated under reduced pressure. In order to remove as much as possible of the inorganic material, the residue was repeatedly dissolved in absolute alcohol and to the solution a little dry ether was added. The solution then was distilled under reduced pressure from a short 45 cc. Claisen distilling flask; the 5,6-dihydroxyhexanone-2 distilled at 120° at 0.3 mm. The total yield was 112 gm. or 70 per cent calcu-

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[3] Traube, W., and Lehmann, E., *Ber. chem. Ges.*, **34**, 1971 (1901).

lated on the basis of the chlorolactone used. The redistilled substance analyzed as follows:

9.285 mg. substance: 18.645 mg. $CO_2$ and 7.505 mg. $H_2O$.
$C_6H_{12}O_3$ (132.13). Calculated. C 54.51, H 9.16.
Found. " 54.75, " 9.04.

*Molecular Weight Determination by Cryoscopy.*—14.196 gm. of water; 0.3676 gm. of substance; 0.371° difference in freezing temperatures.

$$\text{Mol. wt.} = 18.5 \times \frac{100 \times 0.3676}{14.196 \times 0.371} = 129. \quad \text{Mol. wt. calculated, } 132.13.$$

*Molecular Refraction Determination.*

$n_{25} = 1.4673$; $d_{25} = 1.118$; $[M]_D = 32.81$.
$[M]_D$ calculated for straight chain, 32.967.
$[M]_D$ " " cyclic form, 32.40.
Average, 32.68.

### *Diacetyl-5,6-Dihydroxyhexanone-2* ($CH_3COCH_2CH_2CH(OCOCH_3)$-$CH_2OCOCH_3$).

6 gm. of dihydroxyhexanone (b.p. 132° at 2 mm.) were dissolved in 16 cc. of acetic anhydride to which 16 cc. of dry pyridine had been added. The mixture was allowed to stand overnight. After removal of the solvents the diacetate derivative distilled at 112–114° at 0.6 mm. (8.7 gm.). It was insoluble in water. It analyzed as follows:

7.025 mg. substance: 14.410 mg. $CO_2$ and 4.850 mg. $H_2O$.
$C_{10}H_{16}O_5$ (216.18). Calculated. C 55.53, H 7.46.
Found. " 55.93, " 7.72.

*Acetyl Number.*—To 0.1373 gm. of substance were added 35.0 cc. of 0.1 N NaOH. After allowing the mixture to stand overnight 22.3 cc. of 0.1 N HCl were required for neutralization. Calculated, 519; found, 519.

*Molecular Refraction.*—The value points to an open chain formula.

$n_{25} = 1.4398$; $d_{25} = 1.0952$; $[M]_D = 52.00$.
$[M]_D$ calculated for open chain, 51.696.
$[M]_D$ " " cyclo form, 51.128.

*Semicarbazone of Diacetyldihydroxyhexanone.*—Two structures are theoretically possible for the diacetate, one a cyclic and the other a keto form. The existence of the latter at least in predominating quantity is seen from the formation of the semicarbazone in a good yield. The semicarbazone was prepared in the following way. 0.8 gm. of potassium acetate and 0.8 gm. of semicarbazide hydrochloride were dissolved in 2.3 cc. of water. To the solution was added 1 gm. of the diacetate and about 1 cc. of methyl alcohol. The reaction took place with evolution of heat. On standing for 1½ days approximately 1.3 gm. of the crystalline semicarbazone were obtained. On repeated recrystallization from methyl alcohol the substance melted at 112° (with decomposition). It analyzed as follows:

0.5065 mg. substance: 8.975 mg. $CO_2$ and 3.210 mg. $H_2O$.
0.4530 " " : 0.635 cc. N (38°, 752 mm.).
$C_{11}H_{19}O_6N_3$ (273.24). Calculated. C 48.33, H 7.01, N 15.38.
Found. " 48.32, " 7.09, " 15.62.

### *Methylcyclo-5,6-Dihydroxyhexanone-2 (III or IV).*

This substance is readily obtained by dissolving the dihydroxyhexanone in 10 times its weight of dry methyl alcohol containing 0.5 per cent of hydrogen chloride. After 4 hours, the hydrogen chloride was removed with dry silver carbonate. The filtrate was concentrated and the residue distilled at 66–69° at 1.5 mm. In this manner from 10 gm. of dihydroxyhexanone 10 gm. (or 90 per cent) of the methylcyclo derivative were obtained. The analysis was as follows:

5.525 mg. substance: 11.650 mg. $CO_2$ and 4.615 mg. $H_2O$.
0.1752 gm. " : 0.2742 gm. AgI (Zeisel).
$C_7H_{14}O_3$ (146.15). Calculated. C 57.50, H 9.66, $OCH_3$ 21.21.
Found. " 57.50, " 9.34, " 20.66.

The value of molecular refraction was as follows:

$n_{25} = 1.4496$; $d_{25} = 1.0614$; $[M]_D$ calculated 37.13; found 36.97.

*Acetyl Methylcyclo-5, 6-Dihydroxyhexanone-2.*—3 gm. of the methylcyclodihydroxyhexanone were dissolved in a mixture of 9 cc. of acetic anhydride and 9 cc. of dry pyridine. On distillation a fraction was obtained which distilled at 53–54° at 0.1 to 0.2 mm.

Yield 3.3 gm. This material was redistilled and a fraction was collected which boiled at 102–104° at 16 mm. It analyzed as follows:

4.985 mg. substance: 10.580 mg. $CO_2$ and 3.835 mg. $H_2O$.
$C_9H_{16}O_4$ (188.17). Calculated. C 57.42, H 8.57.
Found. " 57.87, " 8.60.

*Acetyl Number.*—0.1666 gm. of substance and 40.0 cc. of 0.1 N NaOH were allowed to stand overnight. 30.4 cc. of 0.1 N HCl were required for neutralization. Calculated, 298; found, 323.

### *Dimethylcyclodihydroxyhexanone.*

18 gm. of methylcyclo-5,6-dihydroxyhexanone-2 were methylated by means of silver oxide and methyl iodide. A total quantity of 50 gm. of silver oxide and 150 gm. of methyl iodide were used and added in four portions. Heating of this reaction mixture was continued for 5 hours. The mixture was then cooled and extracted with dry ether and the ethereal solution dried with anhydrous sodium sulfate. The solvent was removed and three fractions were obtained on distillation.

Fraction I, boiling at 74° at 17 mm., weight 9.4 gm.
" II, " " 75–82° at 17 mm.
" III, viscous residue; weight 1.2 gm.

*Fraction I.*—It had the composition of dimethylcyclodihydroxyhexanone.

5.250 mg. substance: 11.525 mg. $CO_2$ and 4.895 mg. $H_2O$.
0.1076 gm. " : 0.3118 gm. AgI (Zeisel).
$C_8H_{16}O_3$ (160.17). Calculated. C 59.96, H 10.07, $OCH_3$ 38.75.
Found. " 59.85, " 10.43, " 38.25.

The value of the molecular refraction corresponded to the cyclo form.

$n_{25} = 1.4302$; $d_{25} = 0.9922$. $[M]_D^{25}$ calculated for the cyclo form 41.87; found 41.72.

*Fraction III. Condensation Product.*—This fraction represented 1.2 gm. of a viscous residue. It had the composition of an anhydrodihydroxyhexanone.

3.450 mg. substance: 8.005 mg. $CO_2$ and 2.750 mg. $H_2O$.
$C_6H_{10}O_2$ (114.11). Calculated. C 63.12, H 8.83.
Found. " 63.27, " 8.91.

The substance was acetylated by means of acetic anhydride and pyridine. After removal of the acetylation mixture, attempts at fractional distillation under the pressure of about 1 mm. were unsuccessful although the bath was heated to 190°. The substance had the composition of a monoacetylanhydrodihydroxyhexanone.

5.005 mg. substance: 11.245 mg. $CO_2$ and 3.625 mg. $H_2O$.
$C_8H_{12}O_3$ (156.14). Calculated. C 61.51, H 7.75.
Found. " 61.26, " 8.10.

*Acetyl Number.*—0.1960 gm. of substance was refluxed for 1¼ hours with 40.0 cc. of 0.1 N NaOH. 29.0 cc. of 0.1 N HCl were required for neutralization. Calculated, 359; found, 308.

In another methylation experiment 5 gm. of methylcyclodihydroxyketone with 45 gm. of silver oxide and an excess of methyl iodide were refluxed for about 20 hours.

A small amount of a substance was obtained which boiled at 60° at about 17 mm. According to its analysis, it represents monomethoxydihydroxyhexanone anhydride.

5.030 mg. substance: 11.945 mg. $CO_2$ and 4.250 mg. $H_2O$.
0.1806 gm. " : 0.3312 gm. AgI (Zeisel).
$C_7H_{12}O_2$ (128.13). Calculated. C 65.58, H 9.44, $OCH_3$ 24.19.
Found. " 64.75, " 9.45, " 24.20.

### *Condensation of 5,6-Dihydroxyhexanone-2.*

This reaction takes place even at a low temperature. It is probable that a trace of a substance present in the 5,6-dihydroxyhexanone catalyzes this reaction since the yield of the disaccharide on short standing sometimes was higher than when the substance was allowed to stand for a longer period.

Thus, 36 gm. of redistilled 5,6-dihydroxyhexanone-2 were kept at 25° for 12 days and then subjected to distillation. The first fraction consisted of a small quantity of water after which only 24 gm. of substance distilled at the boiling temperature of the dihydroxyketone. It had been also observed that during the distillation further condensation, to a small extent, had taken place. The residue was distilled at about 1 mm. pressure. A fraction was obtained which distilled at 175–180° at 0.3 to 0.4 mm.

The substance had the composition of a dihydroxyhexanonyl dihydroxyhexanone.

6.355 mg. substance: 13.620 mg. $CO_2$ and 5.150 mg. $H_2O$.
$C_{12}H_{22}O_5$ (246.26). Calculated. C 58.50, H 9.01.
Found. " 58.44, " 9.06.

*Acetylation of Distillation Residue.*—In another experiment 31 gm. of 5,6-dihydroxyhexanone-2 were kept at room temperature. This time only one-half of the amount of dihydroxyhexanone was recovered by distillation. 5 gm. of the residue were dissolved in a mixture of 15 cc. of acetic anhydride and 15 cc. of dry pyridine. The following day the reaction product was fractionated. The fraction boiling from 170–180° at 0.2 to 0.3 mm. had the composition of a diacetyl di-(dihydroxyhexanone) anhydride.

3.700 mg. substance: 7.945 mg. $CO_2$ and 2.595 mg. $H_2O$.
$C_{16}H_{26}O_7$ (330.29). Calculated. C 58.15, H 7.94.
Found. " 58.55, " 7.84.

*Acetyl Number.*—0.1318 gm. of substance was refluxed for 2 hours with 40.0 cc. of 0.1 N NaOH. 31.7 cc. of 0.1 N HCl were required for neutralization. Calculated, 340; found, 353.

In another experiment freshly distilled dihydroxyketone was allowed to stand at about 0° for 14 days. On being distilled, only 84 per cent of the original substance was recovered. In order to prevent condensation on standing it is therefore advisable to preserve the dihydroxyketone in a solvent.

### *Methylcyclodihydroxyhexanone from Di-(Dihydroxyhexanone) Anhydride.*

1.7 gm. of the disaccharide were dissolved in 12.5 cc. of methyl alcohol containing 0.5 per cent of hydrogen chloride. After allowing the mixture to stand at room temperature it was neutralized with silver carbonate. The filtered solution was concentrated at a low temperature. At the temperature of 101–103° (15 mm.) a fraction of 1.2 gm. was obtained which had the composition of methylcyclodihydroxyhexanone.

3.290 mg. substance: 6.990 mg. $CO_2$ and 2.980 mg. $H_2O$.
0.0964 gm. " : 0.1554 gm. AgI (Zeisel).
$C_7H_{14}O_3$ (146.15). Calculated. C 57.50, H 9.66, $OCH_3$ 21.21.
Found. " 57.93, " 10.12, " 21.26.

### *Polymerization Product Obtained from 5,6-Dihydroxyhexanone-2 with Concentrated Sulfuric Acid. (According to Hibbert and Timm[2]).*

To 9 gm. of 5,6-dihydroxyhexanone-2 was added 0.009 gm. of concentrated sulfuric acid. The mixture was stirred and heated at 155° for 4 minutes. The dark, resinous material was then kept at 100° under slightly reduced pressure. The substance had the composition of an anhydride of 5,6-dihydroxyhexanone-2.

4.760 mg. substance: 11.075 mg. $CO_2$ and 3.840 mg. $H_2O$.
$(C_6H_{10}O_2)_x$ (114.11). Calculated. C 63.12, H 8.83.
Found. " 63.45, " 9.02.

*Acetylation of Polymeric Anhydride of Dihydroxyhexanone.*—2.1 gm. of the above polymeric anhydride of the dihydroxyhexanone were dissolved in 4.5 cc. of dry pyridine. To this solution 4 cc. of acetic anhydride were added, some heat being evolved. After 36 hours standing the mixture was taken up in chloroform, water was added, and the chloroform solution was washed with dilute solutions of sulfuric acid and sodium carbonate. After drying of the washed chloroform solution with sodium sulfate, the solvent was evaporated.

An attempt was made to distil the residue under reduced pressure but no distillate was obtained. The new compound had the composition of a monoacetate of a polymer dihydroxyhexanone anhydride.

3.600 mg. substance: 8.125 mg. $CO_2$ and 2.410 mg. $H_2O$.
$(C_8H_{12}O_3)_x$ (156.14). Calculated. C 61.51, H 7.75.
Found. " 61.54, " 7.49.

*Acetyl Number.*—0.2031 gm. of substance required 13.7 cc. of 0.1 N NaOH for neutralization. Calculated, 359; found, 378.

### *Anhydride of 5,6-Dihydroxyhexanone-2.*

40 gm. of 5,6-dihydroxyhexanone-2 were heated in a double neck distilling flask at a temperature of 150° for 9 hours. The heating was carried out for 2 to 3 hours at a time, the material being kept in the ice box in between times, about 20 hours. The reaction mixture was then distilled under a pressure of less than 1 mm. The receiving flask was water-cooled, but the vapor leading to the

vacuum pump was passed through a U-tube which was cooled to a very low temperature with solid $CO_2$ and alcohol. On gradually raising the bath temperature, a crystalline material was soon observed in the cold U-tube. It consisted of a mixture of water and anhydride. The temperature of the bath was finally raised to about 160° when most of the material had distilled over. In the first, the water-cooled receiver, a mixture of substances was obtained. The crystalline material in the carbon dioxide-cooled flask was immediately dissolved in dry ether and separated from a small aqueous layer which was formed occasionally. The ether solution was dried with sodium sulfate and kept overnight at 0°. After removal of the ether from the filtered solution the remaining mobile liquid was fractionated. In some instances the non-fractionated material was used. The substance had the composition of an anhydride of 5,6-dihydroxyhexanone-2. On fractionation a portion was obtained which boiled at 40° at 20 mm. or at 55° at 42 mm. This substance had the following composition.

3.860 mg. substance: 8.980 mg. $CO_2$ and 2.990 mg. $H_2O$.

| | | |
|---|---|---|
| $C_6H_{10}O_2$ (114.11). | Calculated. | C 63.12, H 8.83. |
| | Found. | " 63.44, " 8.55. |

*Molecular Weight Determination by Cryoscopy.*—This determination was carried out on a non-fractionated material. 8.093 gm. of benzene; 0.1025 gm. of substance; 0.532° difference of freezing temperatures. Mol. wt. of the monomolecular form ($C_6H_{10}O_2$) calculated, 114.1; found, 121.7.

*Molecular Refraction.*—The molecular refraction as well as the parachor were determined on a pure freshly distilled sample of 5,6-dihydroxycyclohexanone anhydride. The values obtained from determination of the molecular refraction agree with that calculated for a molecule containing two oxygen bridges.

$n_{25} = 1.4350$; $d_{25} = 1.0423$; $[M]_D = 28.57$.

$[M]_D$ calculated (formula VII) 28.79, (formula IX or X) 30.41.

*Determination of Parachor.*—The values found for the distilled anhydride are also in better agreement with a molecule containing two oxygen bridges (formula VII) than with a molecule containing a double bond and one oxygen bridge (formulas IX or X).

The parachor was determined[4] according to Sugden.[5] The calculations were made using his simplified formula:

$$\text{Parachor} = \frac{M}{D}\gamma^{\frac{1}{4}}$$

where $M$ is molecular weight, $D$ density, and $\gamma$ surface tension. The constant of the surface tension apparatus was established by determining the surface tension of known substances. The constant of the apparatus was $0.00380_4$. The parachor of ether, chloroform, and carbon tetrachloride were 209.5, 183.2, and 220 respectively. Calculated 210.2, 184.8, and 222.0 respectively.

Difference of pressure observed on water gauge = 8245 dynes per $cm.^2$
$\gamma_{25} = 31.58$. Parachor found = 259.2.
Calculated, formula VII 254.4; formula IX 271.5; formula X 269.1.

Occasionally, the ethereal extract of the distillate in the carbon dioxide-alcohol-cooled tube turned viscous so rapidly on evaporation of the ether that no appreciable amount of the monomolecular anhydride could be obtained.

### *Acetate of Anhydride of 5,6-Dihydroxyhexanone-2.*

3 gm. of non-fractionated anhydride of 5,6-dihydroxyhexanone-2 were dissolved in a mixture of 7 cc. of pyridine and 6 cc. of acetic anhydride. After standing for 20 hours the mixture was concentrated under reduced pressure. The residue was taken up in chloroform and washed with dilute solutions of sulfuric acid, sodium carbonate, and finally with water. The dried chloroform solution was concentrated and 2 gm. of a substance boiling at 58° at 2 mm. pressure were obtained. The substance had the composition of a monoacetate of an anhydride of dihydroxyhexanone.

5.330 mg. substance: 12.000 mg. $CO_2$ and 3.900 mg. $H_2O$.
$C_8H_{12}O_3$ (156.14). Calculated. C 61.51, H 7.75.
Found. " 61.39, " 8.19.

*Acetyl Number.*—0.0925 gm. of substance required 5.8 cc. of 0.1 N NaOH for neutralization. Calculated, 359; found, 352.

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[4] All the parachor determinations reported in this paper were carried out by Dr. Alexandre Rothen.

[5] Sugden, S., *J. Chem. Soc.*, **125**, 1177 (1924).

The values of the molecular refraction and of the parachor of the substance were in agreement with those calculated for the acetate of anhydrodihydroxyhexanone (acetate of substance IX or X).

*Molecular Refraction.*

$n_{25}$ = 1.4490; $d_4^{25}$ = 1.0575; calculated $[M]_D$ 39.77; found $[M]_D$ 39.55.

*Parachor.*—Difference of pressure observed on water gauge = 8490 dynes per cm².

$\gamma_{25}$ (surface tension) = 32.50; $d_4^{25}$ = 1.0575.

Parachor found = 352.6; parachor calculated = 352.9 (6-membered ring).
" " = 355.3 (5- " " ).

*Hydrogenation of Acetate of Anhydride of 5,6-Dihydroxyhexanone-2.*

0.05 gm. of platinum dioxide (Adams' catalyst) and 4 cc. of 80 per cent alcohol were placed in a small hydrogenation vessel. After reduction of the catalyst, 1 gm. of the above acetyl derivative dissolved in 7 cc. of alcohol was added. A rapid absorption of 135 cc. of hydrogen took place. Further shaking did not increase the absorption of hydrogen. The filtrate from the catalyst was freed from its solvent by distillation, the residue was transferred into a 1.5 cc. double neck distilling flask. 0.5 cc. of a substance distilled at 95° (12 mm.). It had the composition of a dihydro derivative of the acetylated anhydride.

4.065 mg. substance: 9.050 mg. $CO_2$ and 3.300 mg. $H_2O$.
$C_8H_{14}O_3$ (158.15). Calculated. C 60.73, H 8.92.
Found. " 60.71, " 9.08.

*Acetyl Number.*—0.1009 gm. of substance required 6.3 cc. of 0.1 N NaOH for neutralization. Calculated, 355; found, 350.

*Products Obtained in the Water-Cooled Receiver.*

In the water-cooled receiving flask (see anhydride of 5,6-dihydroxyhexanone-2) two substances were identified after acetylation, namely dihydroxyhexanone anhydride of type IX or X and 5,6-dihydroxyhexanone-2. The material separated at times into two layers. The top layer was decanted and acetylated with pyridine and acetic anhydride. It yielded an anhydrodihydroxyhexanone acetate boiling at 50–53° at 1 mm.

5.246 mg. substance: 11.740 mg. $CO_2$ and 3.615 mg. $H_2O$.
$C_8H_{12}O_3$ (156.14). Calculated. C 61.51, H 7.75.
Found. " 61.03, " 7.71.

*Acetyl Number.*—0.0910 gm. of substance required 6.2 cc. of 0.1 N NaOH for neutralization. Calculated, 359; found, 382.

There was a certain amount of a higher boiling fraction which distilled over a wide temperature range.

The bottom layer was also acetylated with pyridine and acetic anhydride. The main fraction distilled at 105° and 0.8 mm. It had the composition of dihydroxyhexanonediacetate.

4.550 mg. substance: 9.420 mg. $CO_2$ and 3.130 mg. $H_2O$.

| | | |
|---|---|---|
| $C_{10}H_{16}O_5$ (216.18). | Calculated. | C 55.53, H 7.46. |
| | Found. | " 56.45, " 7.69. |

*Acetyl Number.*—0.1983 gm. of substance required 16.8 cc. of 0.1 N NaOH for neutralization. Calculated, 519; found, 475.

### *Polymerized Anhydro-5, 6-Dihydroxyhexanone-2.*

20 gm. of 5,6-dihydroxyhexanone-2 were heated in a manner similar to that previously described (three times for 3½ hours at approximately 150°). On distillation of this substance at the bath temperature of 230° under reduced pressure only water came over. The residue was a hard brown resin and had a composition approximating that of a polymerized 5,6-dihydroxyhexanone anhydride.

4.440 mg. substance: 10.445 mg. $CO_2$ and 3.565 mg. $H_2O$.

| | | |
|---|---|---|
| $(C_6H_{10}O_2)_x$ (114.11). | Calculated. | C 63.12, H 8.83. |
| | Found. | " 64.15, " 8.98. |

*Acetate of Polymerized 5,6-Dihydroxyhexanone Anhydride.*—5 gm. of the polymerized anhydride just described were acetylated with a mixture of 11 cc. of dry pyridine and 8 cc. of acetic anhydride. The following day the mixture was taken up in 100 cc. of chloroform, washed with dilute solutions of sulfuric acid and sodium carbonate and finally with water. On evaporation of the solvent and distillation of the residue a very small fraction was obtained which had the character of the anhydride of the 5,6-dihydroxyhexanone-2. The main portion could not be distilled even under greatly reduced pressure. It had the approximate composition of the acetate of the anhydrodihydroxyhexanone.

| | | |
|---|---|---|
| $(C_8H_{12}O_3)_x$ (156.14). | Calculated. | C 61.51, H 7.75. |
| | Found. | " 62.05, " 7.48. |

*Acetyl Number.*—0.1554 gm. of substance required 11.1 cc. of 0.1 N NaOH for neutralization. Calculated, 359; found, 400.

*Hydrogenation of Acetate of Polymerized Dihydroxyhexanone Anhydride.*—0.05 gm. of Adams' platinum dioxide catalyst was reduced in an alcoholic medium and 1 gm. of the above polymerized anhydride acetate was then added. Within 4 hours 20 cc. of hydrogen were absorbed. Further shaking did not increase the absorption although the theory required 141 cc.

### *Treatment of Polymerized 5,6-Dihydroxyhexanone Anhydride with Methyl Alcohol Containing Hydrogen Chloride.*

If the polymerized anhydride was treated with cold methyl alcohol containing 0.5 per cent of hydrogen chloride, the material remained practically unchanged. If, on the other hand, it was refluxed with the same amount of the solvent, a semisolid substance was obtained which contained methoxyl groups. Thus, 5 gm. of polymerized anhydride were refluxed with 50 cc. of methyl alcohol containing 0.5 per cent of hydrogen chloride for 4 hours. The following morning the hydrogen chloride was removed with silver carbonate and the methyl alcohol evaporated under reduced pressure. Nothing distilled at the bath temperature of 170° the pressure being less than 1 mm. The composition of the substance was approximately that of a compound consisting of five dihydroxyhexanone rests containing one methyl group.

5.320 mg. substance: 12.090 mg. $CO_2$ and 4.190 mg. $H_2O$.
14.485 " " : 5.845 " AgI (Zeisel).
$C_{31}H_{54}O_{11}$. Calculated. C 61.76, H 9.03, $OCH_3$ 5.15.
Found. " 61.97, " 8.81, " 5.32.

### *Distilled Polymeric Anhydride of Dihydroxyhexanone.*

If the solid polymeric anhydride was heated in a bath at a temperature of 230–290° and under highly reduced pressure, a small amount of a yellow viscous material distilled at 130–180° at 0.5 mm. It had the composition of a dihydroxyhexanone anhydride.

3.815 mg. substance: 8.780 mg. $CO_2$ and 3.055 mg. $H_2O$.
$(C_6H_{10}O_2)_x$ (114.11). Calculated. C 63.12, H 8.83.
Found. " 62.76, " 8.96.

*Acetylation of Distilled Polymeric Anhydride of Dihydroxyhexanone.*—1.5 gm. of the above substance were acetylated with a mixture of 3 cc. of pyridine and 3 cc. of acetic anhydride. After standing for 15 hours, the mixture was taken up in chloroform, washed with dilute solutions of sulfuric acid and sodium carbonate and finally with water. After drying the solvent was removed by distillation; the residue distilled approximately at the temperature of the boiling point of the acetate of the monomeric dihydroxyhexanone anhydride. It was not analyzed. A second fraction was obtained which boiled at 92–125° at 0.8 mm. It had the following composition: C 60.6; H 8.31; acetyl number 267. The remaining 0.7 gm. of light brown viscous material had practically the composition of an acetate of dihydroxyhexanone anhydride.

3.610 mg. substance, 8.090 mg. $CO_2$ and 2.610 mg. $H_2O$.
$(C_8H_{12}O_3)_x$ (156.14). Calculated. C 61.51, H 7.75.
Found. " 61.11, " 8.08.

*Acetyl Number.*—0.1099 gm. of substance required 6.2 cc. of 0.1 N NaOH for neutralization. Calculated, 359; found, 319.

The brown distillation residue obtained from the fraction boiling at 130–180° at 0.5 mm. possessed the composition of C 65.55, and H 9.06.

### *Hydrogenation of 5,6-Dihydroxyhexanone-2.*

7 gm. of freshly distilled 5,6-dihydroxyhexanone-2 were dissolved in 40 cc. of glacial acetic acid. To this solution was added 0.12 gm. of Adams' platinum dioxide catalyst. After elimination of the air in the flask, a pressure of 1.5 atmospheres of hydrogen was applied. In 4 hours approximately 1800 cc. of hydrogen were absorbed. After filtering off the catalyst most of the acetic acid was evaporated under diminished pressure. The residue was taken up in ether and the mixture was washed with a dilute solution of sodium carbonate. The dried ether solution then was fractionated and 3 gm. of a fraction boiling at 70–73° at 14 mm. were obtained. The substance did not reduce Fehling's solution nor did it form a semicarbazone. It had the composition of a monohydroxyhexanone and has the structure of a tetrahydrofuran or tetrahydropyran derivative (formula XI or XII). The analysis was as follows:

3.775 mg. substance: 8.600 mg. $CO_2$ and 3.495 mg. $H_2O$.
$C_6H_{12}O_2$ (116.13). Calculated. C 62.02, H 10.42.
Found. " 62.12, " 10.36.

*Acetate of Monohydroxy Derivative.*—To 2.6 gm. of the above substance was added a mixture of 5 cc. of pyridine and 5 cc. of acetic anhydride. The mixture was allowed to stand for 5 hours and was then concentrated under reduced pressure. The residue was taken up in chloroform and the solution was washed with dilute sulfuric acid and sodium carbonate. The dried chloroform solution gave on fractionation 2 cc. of a substance which boiled at 88° at 13 mm. The substance possessed the composition of a monoacetyl derivative.

5.350 mg. substance: 11.955 mg. $CO_2$ and 4.235 mg. $H_2O$.
$C_8H_{14}O_3$ (158.15). Calculated. C 60.73, H 8.92.
Found. " 60.93, " 8.85.

*Acetyl Number.*—0.1072 gm. of substance required 6.8 cc. of 0.1 N NaOH for neutralization. Calculated, 355; found, 356.

*Molecular Refraction.*—The value of the molecular refraction of the substance was in agreement with its cyclic structure.

$d^{25} = 1.0175$; $n^{25} = 1.4321$; $[M]_D = 40.32$.
$[M]_D$ calculated 40.24 (cyclic form).
$[M]_D$ " 40.81 (open chain).

# ON THE RING STRUCTURE OF METHYLGLUCODESOSIDE.

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The great instability of the ribodesosenucleosides as well as that of ordinary glucosides of 2-desoxy sugars made it necessary to investigate into the ring structure of these glucosides inasmuch as in regard to stability they resembled very much the $\gamma$-glucosides having the $<1,4>$ structure. It is natural that the same question should also have interested Bergmann and while the present investigation was in progress there appeared a paper by Bergmann and Breuers dealing with this problem.[1] These authors prepared 4-glucosidoglucodesose from cellobiose. This disaccharide could not form a $<1,4>$ glucoside and yet the methylglucoside prepared by them was characterized by a great instability. Thus, they attributed to the common methylglucodesoside the $<1,5>$ ring structure. However, to make their proof really rigorous, they should have demonstrated that methylglucodesoside prepared from the disaccharide was identical with the glucoside prepared directly from the glucodesose. In view of this fact our work was brought to a conclusion and decisive evidence was furnished for the $<1,5>$ structure of the methylglucodesoside. Our conclusion is based on the following facts.

Glucodesose was oxidized to the acid, which was converted into the lactone and the lactone was methylated. On the other hand, methylglucodesoside was exhaustively methylated. The methylated glucoside was converted into the corresponding sugar which was oxidized to the corresponding methylated sugar acid and this transformed into the corresponding lactone. In this

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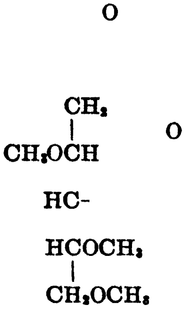
[1] Bergmann, M., and Breuers, W., *Ann. Chem.*, **470**, 51 (1929).

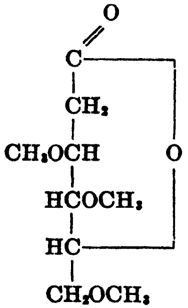
way two trimethyl glucodesonic lactones were obtained, one starting from glucodesonic acid and the other from methylglucodesoside. To the first lactone, structure I may be attributed, by analogy with other sugar acids.



<1,4> lactone.

Crystalline.

I.



<1,5> lactone.

Liquid.

II.

The conclusion regarding the ring structure of the second lactone depends upon its identity or non-identity with the former. The first lactone crystallized readily. It was practically inactive in chloroform solution and had a rotation of $[\alpha]_D^{25} = +21.5°$ in benzene. The second lactone was a liquid and had a rotation of $[\alpha]_D^{25} = +87.5°$ in chloroform and of $[\alpha]_D^{29} = +88.2°$ in benzene. Hence the two lactones are not identical and it is warranted to assign the <1,5> ring structure to the second one and on the basis of this, also to the parent glucoside. *Thus the great instability of the 2-desoxyglucoside is due not to a <1,4> ring structure but to the reduced state of carbon atom 2.*

## EXPERIMENTAL.

*2-Desoxy-d-Gluconic Acid.*—The oxidation of 2-desoxy-*d*-glucose[2] was carried out following the method of Goebel.[3] The solutions used were prepared as follows: Solution A, 76 gm. of finely pulverized iodine were thoroughly mixed with 150 gm. of barium iodide,

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[2] Bergmann, M., Schotte, H., and Lechinsky, W., *Ber. chem. Ges.*, **55**, 165 (1922).

[3] Goebel, W. F., *J. Biol. Chem.*, **72**, 801 (1927).

1900 cc. of water were added, and the mixture was shaken until all the iodine and barium iodide were dissolved; Solution B, 124 gm. of $Ba(OH)_2 \cdot 8\ H_2O$ were dissolved in 1900 cc. of water.

The oxidation of the sugar was carried out as follows: 4 gm. of 2-desoxyglucose were dissolved in 322 cc. of Solution A. The mixture was cooled to $-5°$ and 487 cc. of Solution B were added from a dropping funnel in the course of 10 minutes with vigorous stirring. During the addition of the hydroxide the temperature was maintained at $-5°$. When all the barium hydroxide was added, the reaction was allowed to proceed for 5 more minutes. 22 gm. of lead carbonate were then added, and then 52 cc. of 25 per cent sulfuric acid. After a few minutes of stirring, enough lead carbonate was added to render the solution neutral to Congo red. The lead iodide was then filtered off and the filtrate concentrated to a small volume under reduced pressure. The last traces of hydroiodic acid were removed by treating the solution with a small excess of silver sulfate. Silver and lead were then removed with hydrogen sulfide. The solution was finally treated with an excess of barium carbonate and filtered. On concentration of the filtrate, the barium salt separated in crystalline form, and was filtered off. A second crop of salt was obtained from the filtrate by pouring it into a fairly large volume of methyl alcohol. The total yield of the Ba salt was 6.14 gm. It analyzed as follows:

4.485 mg. substance: 4.790 mg. $CO_2$ and 1.775 mg. $H_2O$.
6.470 " " : 3.055 " $BaSO_4$.
$C_{12}H_{22}O_{12}Ba$. Calculated. C 29.06, H 4.44, Ba 27.88.
Found. " 29.12, " 4.42, " 27.80.

In order to obtain the free acid, the salt was dissolved in water and the barium quantitatively removed with sulfuric acid. On concentration under reduced pressure the acid separated in crystalline form. It melted at 145° (uncorrected) and analyzed as follows:

5.705 mg. substance: 8.250 mg. $CO_2$ and 3.260 mg. $H_2O$.
$C_6H_{12}O_6$ Calculated. C 40.00, H 6.66.
Found. " 39.92, " 6.65.

The pure substance showed a rotation of

$$^{25}\quad \frac{+0.15° \times 100}{1 \times 6.288} = +2.4° \text{ (in water).}$$

1 hour later the rotation was

$$[\alpha]_D^{25} = \frac{+0.22° \times 100}{1 \times 6.288} = +3.5°.$$

3 days later it was

$$[\alpha]_D^{25} = \frac{+2.09° \times 100}{1 \times 6.288} = +33.2°.$$

At the end of that time equilibrium had not been reached.

*Glucodesonic Lactone.*—As was to be expected, it was found that the rate of lactone formation was accelerated by raising the temperature. It was therefore found expedient to make a 2 per cent solution of the acid and to heat it in sealed tubes for 24 hours in a steam-heated water bath at 100°. The solution was then concentrated to nearly dryness under reduced pressure and in order to remove as much water as possible, the residue was taken up in benzene and the latter removed by distillation under reduced pressure, the operation being repeated several times. On standing, beautiful colorless needles settled out on the walls of the flask. The contents of the flask were then dissolved in a large excess of acetone. Only a small part remained insoluble. As a rule, 2.0 gm. of the acid were used for each experiment. For the extraction with acetone several experiments were combined. The hot acetone extract was allowed to concentrate in a vacuum desiccator. The first crystals which formed on standing were composed in the main of the acid. From the mother liquor the lactone crystallized in heavy crystals, about four or five crops of crystals being obtained. For final purification it required five recrystallizations. Only then did the substance acquire a rather sharp melting point of 95–97° (uncorrected). The composition of the substance was the following.

4.475 mg. substance: 7.310 mg. $CO_2$ and 2.575 mg. $H_2O$.

| | | |
|---|---|---|
| $C_6H_{10}O_5$. | Calculated. | C 44.42, H 6.21. |
| | Found. | " 44.54, " 6.19. |

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+1.36° \times 100}{1 \times 2} = +68.0° \text{ (in water)}.$$

*Trimethyl-d-glucodesonic Lactone <1,4>*.—The above lactone was methylated once with methyl iodide and silver oxide first in acetonic solution and the product of this methylation remethylated directly in methyl iodide solution. 4.0 gm. of the crystalline lactone were dissolved in acetone and methylated with an excess of methyl iodide. The product of the second methylation was concentrated to a thick syrup under diminished pressure. The residue was taken up in very little ether and was allowed to stand in a desiccator at 10°. After several hours the substance turned into a crystalline mass. The substance was recrystallized from ether. It contracted at 59° and melted at 62° (uncorrected). It had the following composition.

4.710 mg. substance: 9.175 mg. $CO_2$ and 3.315 mg. $H_2O$.
4.280 " " : 14.75 " AgI (Zeisel).
$C_9H_{16}O_5$. Calculated. C 52.90, H 7.80, $OCH_3$ 45.55.
Found. " 53.12, " 7.87, " 45.34.

There was no decisive rotation in chloroform.

The rotation was

$$[\alpha]_D^{25} = \frac{+0.43^\circ \times 100}{1 \times 2} = +21.5^\circ \text{ (in benzene).}$$

*Trimethyl-d-Methyglucodesoside.*—The methyl 2-desoxy-*d*-glucoside used in this experiment was prepared according to the directions of Bergmann and coworkers.[4] Without any purification other than the removal of the hydrochloric acid and the solvent, the glucoside was methylated with dimethyl sulfate and sodium hydroxide in the usual manner. For 10 gm. of the glucoside, 84 cc. of dimethyl sulfate and 84 cc. of 30 per cent sodium hydroxide were used.

The reaction flask was fitted with two graduated funnels, a return condenser, and a mechanical stirrer. 10 gm. of the glucoside were dissolved in 10 cc. of water and transferred into the reaction flask. 10 cc. of sodium hydroxide were then introduced through one of the dropping funnels and the mixture was heated to 50° in a water bath. The dimethyl sulfate and the sodium hydroxide were then allowed to flow at equal rate each from a

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[4] Bergmann, M., Schotte, H., and Lechinsky, W., *Ber. chem. Ges.*, **55**, 169 (1922).

separate funnel into the rapidly stirred solution. Care was taken to keep the solution slightly alkaline throughout the reaction. The water bath was maintained at 50° during the first half of the reaction, and at 60° during the latter half. When all the alkali and dimethyl sulfate had been added, the mixture was heated for another half hour to decompose all the dimethyl sulfate. The glucoside was then extracted with chloroform and the solution was washed free of alkali and dried over sodium sulfate. The chloroform was removed and the glucoside distilled under a pressure of 0.35 mm. It distilled at 86–90°. The rotatory power of the glucoside varied with the different preparations, ranging from $[\alpha]_D^{25} = +64°$ to $[\alpha]_D^{25} = +97°$ (in chloroform). The relative amount of $\alpha$ and $\beta$ forms obviously varied with the various experiments. The pure methylated glucoside analyzed as follows:

5.690 mg. substance: 11.180 mg. $CO_2$ and 4.570 mg. $H_2O$.
0.1053 gm. " : 0.4487 gm. AgI (Zeisel).
$C_{10}H_{20}O_5$. Calculated. C 54.54, H 9.09, $OCH_3$ 56.31.
Found. " 54.34, " 9.01, " 56.24.

*Trimethyl-d-Glucodesose.*—10 gm. of trimethyl-2-desoxymethyl-*d*-glucoside were dissolved in 200 cc. of hot 0.1 N hydrochloric acid and heated for 10 minutes on the steam bath. The solution was cooled, treated with an excess of silver sulfate, and the filtrate freed from sulfuric acid and evaporated to dryness under reduced pressure. The residue was taken up in warm ether and filtered. The filtrate was transferred to a beaker and evaporated to dryness in a vacuum desiccator. The residue, obtained in a good yield, crystallized spontaneously. It was recrystallized from a mixture of ether and petrolic ether. When pure it melted at 58–61° and showed a rotation of

$$[\alpha]_D^{25} = \frac{+2.30° \times 100}{1 \times 3.864} = +59.5° \text{ (in water).}$$

$$[\alpha]_D^{25} = \frac{+1.28° \times 100}{1 \times 3.864} = +33.1° \text{ (at equilibrium in water).}$$

It analyzed as follows:

5.045 mg. substance: 9.705 mg. $CO_2$ and 3.935 mg. $H_2O$.
5.000 " " : 17.110 " AgI (Zeisel).
$C_9H_{18}O_5$. Calculated. C 52.42, H 8.73, $OCH_3$ 45.14.
Found. " 52.45, " 8.72, " 45.17.

*Trimethyl-d-Glucodesonic Acid.*—The procedure for the oxidation of trimethyl-2-desoxy-*d*-glucose was similar to that used for the oxidation of 2-desoxy-*d*-glucose. Slight modifications were made, however: Solution B was added in the course of 5 minutes and the reaction was allowed to proceed for 5 minutes more before the lead carbonate and sulfuric acid were added. The proportions of the various reagents used were exactly the same. The salt, which was obtained as a viscous residue after evaporation to dryness, was taken up with a little dry methyl alcohol and filtered. The filtrate was concentrated and then poured into a mixture of methyl alcohol and ether. The Ba salt separated as a white amorphous powder. It was pure without further treatment as indicated by the analysis:

7.170 mg. substance: 9.680 mg. $CO_2$ and 3.650 mg. $H_2O$.
11.030 " " : 4.530 " $BaSO_4$.
$(C_9H_{17}O_6)_2Ba$. Calculated. C 37.26, H 5.90, Ba 23.70.
Found. " 36.81, " 5.69, " 24.16.

This salt showed the following rotation.

$$[\alpha]_{D}^{25} = \frac{+\ 1.45^\circ \times 100}{2 \times 4.000} = +\ 18.1^\circ \text{ (in water)}.$$

The acid showed the following rotations.

0.0914 gm. of salt was dissolved in 1 equivalent of 0.1 N hydrochloric acid and the solution was diluted to 5 cc. with water.

$$[\alpha]_{D}^{25} = \frac{+\ 0.62^\circ \times 100}{2 \times 1.4014} = +\ 22.1^\circ.$$

0.0756 gm. of salt was dissolved in 1 cc. of 36 per cent hydrochloric acid (excess) and the solution was diluted to 2.5 cc. with water.

$$[\alpha]_{D}^{25} = \frac{+\ 1.34^\circ \times 100}{1 \times 2.3184} = +\ 57.8^\circ.$$

*Trimethyl-d-Glucodesonic Lactone <1,5>.*—The above barium salt was dissolved in water and the barium removed quantitatively. The solution was then concentrated under reduced pressure. The residue did not dissolve completely in ether; hence it was taken up in acetone and concentrated at 0.02 mm. pressure, the temperature of the bath being 80°. This residue analyzed fairly well for the lactone ($C = 52.81$ and $H = 7.48$). The substance was then taken up in ether and the solution filtered from a slight insoluble precipitate. The ether was then removed by distillation and the residue distilled. It boiled at 137° under a pressure of 0.02 mm. This boiling point, however, is not regarded as definitely correct in view of the small quantity of material available. The distillate analyzed correctly for the lactone.

4.870 mg. substance: 9.470 mg. $CO_2$ and 3.500 mg. $H_2O$.
$C_9H_{16}O_6$. Calculated. C 52.90, H 7.80.
Found. " 53.02, " 8.04.

The rotation of the substance was as follows:

$$[\alpha]_D^{25} = \frac{+3.5° \times 100}{1 \times 4} = +87.5° \text{ (in chloroform).}$$

$$\frac{+0.90° \times 100}{1 \times 1.02} = +88.2° \text{ (in benzene).}$$

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